The effects of breakfast and exercise for postprandial metabolism and energy balance

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Award date:
2019

Awarding institution:
University of Bath

THE EFFECTS OF BREAKFAST AND EXERCISE FOR POSTPRANDIAL METABOLISM AND ENERGY BALANCE

Volume 1 of 1

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A thesis submitted for the degree of Doctor of Philosophy
University of Bath
Department for Health
August 2019

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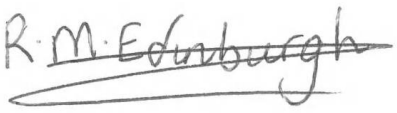
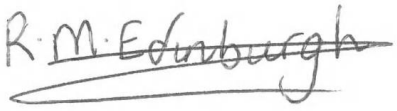
Candidate Signature: 

Declaration of authorship:
I am the author of this thesis, and the work described therein was carried out by myself personally, with the exception of:

In **Chapters 3 and 5**, some experimental work (the analysis of blood samples using gas-chromatography mass-spectrometry) was completed with the help of researchers at the University of Birmingham. In **Chapter 6**, aspects of the experimental work relating to the '*Training Study*' (the analysis of the phospholipid composition of skeletal muscle samples) was completed at the Medical University of Białystok. In addition, in **Chapter 6**, all data relating to the '*Acute Study*' was collected at the University of Birmingham.

Candidate Signature: 

THE EFFECTS OF BREAKFAST AND EXERCISE FOR POSTPRANDIAL METABOLISM AND ENERGY BALANCE

ACKNOWLEDGMENTS 6

THESIS ABSTRACT 7

THESIS LAY SUMMARY 8

RELEVANT PUBLICATIONS 11

COMMON ABBREVATIONS USED 12

CHAPTER 1 - LITERATURE REVIEW 13

OBESITY AND METABOLIC DISEASE 13

FASTING AND POSTPRANDIAL METABOLISM 14
Glucose metabolism 14
Fatty acid metabolism 20
The glucose-fatty acid cycle (Randle cycle) 23

METABOLIC FLEXIBILITY AND INSULIN SENSITIVITY 32
Impaired fasting metabolism 32
Impaired postprandial metabolism 33
Lipid metabolism and insulin resistance 35

LIFESTYLE MODIFICATIONS 42
Dietary modifications 42
Exercise 44

SUBSTRATE METABOLISM DURING EXERCISE 49
Limitations to lipid utilisation 52
Variation in lipid utilisation during exercise 53

BREAKFAST, EXERCISE AND GLYCAEMIA 56
Glycaemia during- and post-exercise (acute) 56
Exercise training and glycaemia 58

BREAKFAST, EXERCISE AND ENERGY BALANCE 67

CONCLUDING STATEMENTS 72

CHAPTER 2 - GENERAL METHODS 74

PARTICIPANTS 74

ANTHROPOMETRY **75**

STANDARDISATION PROTOCOLS **76**

EXPIRED GAS SAMPLES **77**
Continuous breath-by-breath gas analysis 78
Douglas bag analysis 79

GLUCOSE TOLERANCE AND INSULIN SENSITIVITY **82**

BLOOD SAMPLING AND TREATMENT **84**
Automatic analyser (Daytona) 85
Enzymatic colorimetric assays 86
Enzyme-linked immunoassays (ELISA) 86
Precision of analysis 88

BLOOD GLUCOSE FLUX **90**
Preparation of tracers 92
Sample preparation 93
Gas chromatography mass spectrometry (GC-MS) 93
Data modelling and calculations 94

MUSCLE SAMPLING AND ANALYSIS **96**
Powdering 97
Assessment of protein content 97
Western blots 98

ENERGY BALANCE **99**
Assessing energy intake 99
Assessing energy expenditure 103

STATISTICAL ANALYSIS **108**

CHAPTER 3 - BREAKFAST-EXERCISE & GLYCAEMIA **112**

RELEVANCE TO THESIS **113**

ABSTRACT **115**

INTRODUCTION **116**

METHODS **117**

RESULTS **129**

DISCUSSION **142**

REFERENCES FOR CHAPTER 3 ..... **148**

CHAPTER 4 - BLOOD SAMPLING METHODS **152**

RELEVANCE TO THESIS....153
ABSTRACT....159
INTRODUCTION....160
METHODS....161
RESULTS....166
DISCUSSION....172
REFERENCES FOR CHAPTER 4....175
CHAPTER 5 - BREAKFAST-EXERCISE & ENERGY BALANCE....183
RELEVANCE TO THESIS....184
ABSTRACT....195
INTRODUCTION....196
METHODS....198
RESULTS....205
DISCUSSION....210
REFERENCES FOR CHAPTER 5....215
CHAPTER 6 - BREAKFAST-EXERCISE TRAINING STUDY....218
RELEVANCE TO THESIS....219
INTRODUCTION....223
METHODS....225
RESULTS....240
DISCUSSION....258
REFERENCES FOR CHAPTER 6....264
CHAPTER 7 - GENERAL DISCUSSION....270

ACKNOWLEDGMENTS

I would like to thank Dr Javier Gonzalez for being a faultless primary supervisor and for guiding me through this PhD. During my time at the University of Bath, first as an undergraduate and then as a postgraduate student, you have not only been a fantastic supervisor, but a great friend and have had a huge impact on my personal and academic development. Your passion for science, work ethic and positive outlook even if things go a bit wrong have been a source of inspiration and I have enjoyed every day as a member of 'Team Gonzalez'. I look forward to seeing you (continue to) become a world-leading academic. I would like to extend my gratitude to Professors Dylan Thompson and James Betts for your invaluable contributions. Thanks also go to Dr. Francoise Koumanov for your support and patience, in particular when doing muscle analysis for the studies in this thesis. You have been an excellent teacher and I will always be so grateful. Whilst I don't have the space to acknowledge all the researchers I would like to, special thanks also go to Dr. Jean-Philippe Walhin, Aaron Hengist and Ollie Chrzanowski-Smith.

Next, to my participants, I would like to say a massive thank you for helping me complete my research and in particular everyone that put up with me during the supervised exercise training sessions. Getting to know you all has been a great experience and my work simply would not have been possible without your help. I would like to also thank the University of Bath, the Rank Prize Funds and the Physiological Society who have funded my research over the past 4 years.

On a personal note, a huge thank you goes to Helen, for being a continual source of support and for putting up with all my early starts (you can blame Javi for that) and ridiculous bed times. I have loved every moment of our time together in Bath and I can't wait for future adventures together. I would like to give special thanks to Sam and Ollie who have been wonderful friends during my time in Bath and to Jamie and Henry and I look forward to seeing your own successes in academia. Finally, I would like to thank my family for their love and support, and in particular Julie Edinburgh, for being a continual source of inspiration and great role model.

THESIS ABSTRACT

Whilst exercise training can improve aspects of metabolic health (e.g. increasing insulin sensitivity), there can be some apparent variability between people in their response to supervised exercise training. Exercise studies typically do not control for the nutrient status of participants pre-exercise and this may, at least partially, explain this variable response. Performing a single bout of exercise before *versus* after eating breakfast can alter substrate metabolism during- and energy balance post-exercise. This includes increasing the utilisation of fat as a substrate during exercise, augmenting the activation of intramuscular signalling pathways relating to substrate metabolism and when breakfast is omitted, lowering energy intake on the day that the exercise is performed. These acute exercise-responses may be important for metabolic health when regularly accrued with training, especially for humans with obesity, for whom impairments relating to lipid metabolism and/or a low turnover of endogenous lipid pools (muscle and adipose tissue) may explain the association between obesity, physical inactivity and insulin resistance. The studies in this thesis first showed that when healthy men performed a bout of endurance-type exercise, substrate utilisation during exercise, plasma glucose flux at a meal consumed after the exercise, post-exercise intramuscular signalling responses and 24 h energy balance were dependant on whether breakfast was consumed before the exercise or omitted (**Chapters 3 and 5**). Nutrient-exercise timing was then shown to mediate training responsiveness in men classified as overweight or obese, with exercise before *versus* after breakfast increasing oral glucose insulin sensitivity, which was linked to increased lipid utilisation during exercise and augmented skeletal muscle adaptations (**Chapter 6**). These findings have implications for future research and clinical practice. For example, both acute and training exercise studies should control for nutrient-timing as a potential confounding variable when measures of postprandial metabolism are outcome measures and for populations at risk of metabolic diseases, endurance-type exercise should be recommended before *versus* after nutrient consumption (breakfast) to increase the health benefits acquired from training.

Key Words: exercise; breakfast; metabolism; insulin sensitivity; energy balance.

THESIS LAY SUMMARY

Obesity prevalence in adults from the UK rose from 23 % to 29 % from 2000 to 2016 (1) and similar trends were apparent worldwide, meaning that now ~ 2 billion humans can be classified as overweight (2). Obesity is associated with a higher risk of type 2 diabetes (T2D) and cardiovascular disease (CVD) (3) and incurs costs equating to ~ 3 % of total global spending (4). Bariatric surgery can achieve sustained weight loss and remission from metabolic diseases, but the costs and the risks involved, means that surgery is unlikely to be a solution to obesity at a population level (5). Lifestyle modifications such as exercising more frequently are also recommended to help prevent and treat metabolic diseases and obesity. However, many people fail to achieve current exercise guidelines, partly due to a perceived lack of time (6). For these people and for people who cannot increase the intensity or duration of exercise beyond a limit (e.g. due to a low fitness level), increasing the health benefits from any exercise that is performed is important. In addition, there is evidence of variability between people in their response to the same supervised exercise training, for some important health outcomes (7). The timing of food intake in relation to the exercise may be important in this regard, as exercising before *versus* after eating breakfast alters metabolism during- and after-exercise and some of the adaptive responses in exercised muscle (8).

An infrequent use of fat stores in skeletal muscle and adipose (fat) tissue and/or a low capacity for using fat as a substrate for energy metabolism might, at least partially, explain a link between obesity and metabolic diseases, such as T2D (9). Most health-related research that has studied the effects of consuming breakfast before *versus* after exercise, or exercising and omitting breakfast altogether, has been conducted in a laboratory, in healthy men, and over a short (< 24 h) time frame (8). Exercising before breakfast increases fat use during exercise and also alters the magnitude of some adaptations that are important for health in muscle and fat tissue compared to exercising after- (and particularly a carbohydrate-rich) breakfast (10-14). In theory, this may enhance the health benefits of the exercise. One previous study showed that if healthy men eat a high-calorie and high-fat

diet over six weeks, exercising before, but not after, consuming a carbohydrate-rich breakfast increased estimates of insulin sensitivity (a predictor of T2D risk) compared to a non-exercise group (11). Other research has also shown that the energy deficit created from omitting breakfast before exercise is not compensated for with a higher energy intake after exercise over 24 h (15, 16), which may create the conditions for a negative energy balance and greater long-term weight loss, compared to when breakfast is eaten before exercise. This finding is line with the theory that people who use less carbohydrate and more fat to fuel exercise may be less likely to compensate for the energy deficit from the exercise by eating more afterwards (17). However, an assessment of energy intake *and* expenditure over 24 h has not been reported in this context. Training studies in humans with obesity are also required to develop the current research in healthy men.

As such, the research in this thesis aimed to investigate the effects of breakfast consumption *versus* omission before exercise or altered breakfast timing, for the short- and longer-term regulation of energy metabolism and energy balance. The research in **Chapter 3** showed that glucose metabolism and adaptive responses in skeletal muscle differ after a single bout of exercise performed after breakfast, compared to if breakfast is omitted. Some methodological considerations were then explored in **Chapter 4** and insights were provided into how different methods of sampling blood can alter the concentrations of measured metabolites. Then, in **Chapter 5**, an assessment of all behavioural components of energy balance was completed over 24 h, with exercise and breakfast consumption *versus* omission. In that study, we showed that breakfast omission *versus* consumption before exercise creates a more negative daily energy balance in healthy men, and that rates of carbohydrate use from the liver may help to explain differences in energy intake after exercise. Finally, in **Chapter 6**, thirty middle-aged and sedentary men classified as either overweight or obese, were allocated to either a no-exercise group, a breakfast before exercise group or an exercise before breakfast group for a six-week intervention. The exercise groups completed 30-50 min cycling, three times weekly and ate the same breakfast, but either before or after they did the exercise. Oral glucose insulin sensitivity (a predictor of T2D risk) improved

with exercise training before *versus* after breakfast and this was accompanied by augmented adaptations that relate to metabolic health in the exercised muscle. This response also occurred despite no clear differences between the groups for changes in body mass or behavioural components of energy balance in response to the intervention. That study therefore suggested that for some important health outcomes, people classified as overweight or obese should consider exercising before *versus* after eating breakfast. Overall, the research in this thesis provides insights into how exercise performed during fasting (i.e. with breakfast omission or with breakfast consumption but *after* exercise) alters the short- and the longer-term regulation of energy metabolism and energy balance relative to exercising after eating. In **Chapter 7**, the results from all of the studies and their implications for future research and exercise or nutrition guidelines was discussed in detail.

RELEVANT PUBLICATIONS

Edinburgh, R.M., Betts, J.A., Burns, S.F. & Gonzalez, J.T (2017). Concordant and divergent strategies to improve postprandial glucose and lipid metabolism. *Nutrition Bulletin*, *42* (2), 113-122.

Edinburgh, R.M., Hengist, A., Smith, H.A., Betts, J.A., Thompson, D., Walhin, JP. & Gonzalez, J.T (2017). Prior exercise alters the difference between arterialised and venous glycaemia: implications for blood sampling procedures. *British Journal of Nutrition*, *117* (10), 1114-1121.

Edinburgh, R.M., Betts, J.A., Thompson, D., & Gonzalez, J.T (2017). Physiologists, watch where you place the cannula! *Physiology News*, *109*, 20-23.

Edinburgh, R.M., Hengist, A., Smith, H.A., Travers, R.L., Koumanov, F., Betts, J.A., Thompson, D., Walhin, J.P., Wallis, G.A., Hamilton, D.L., Stevenson, E.J., Tipton, K.D., Gonzalez, J.T. (2018). Pre-exercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. *American Journal of Physiology-Endocrinology and Metabolism*, *315* (5), 1062-1074.

Edinburgh, R.M., Betts, J.A., & Gonzalez, J.T. (2018). Fighting media misreporting. A case study in diet and exercise. *Physiology News*, *113*, 22-23.

Edinburgh, R.M., Hengist, A., Smith, H.A., Travers, R.L., Koumanov, F., Betts, J.A., Thompson, D., Walhin, J.P., Wallis, G.A., Hamilton, D.L., Stevenson, E.J., Tipton, K.D., Gonzalez, J.T. (2019). Skipping breakfast before exercise creates a more negative 24-h energy balance: A randomized controlled trial in healthy physically active young men. *Journal of Nutrition*, *149* (8), 1326-1334.

COMMON ABBREVATIONS USED

Throughout this thesis abbreviations are defined upon first use in each chapter.

This list only details the abbreviations that are most commonly used:

Akt = protein kinase B (also known as PKB)
AMPK = 5′- adenosine monophosphate activated protein kinase
AS160 = Akt substrate of 160 kDa
AUC = area under the curve (**iAUC** = incremental area under the curve)
AV = arterialised-venous
BE (or BR-EX) = breakfast before exercise
BHB = *β*-hydroxybuturate
***β*-HAD =** *β*-hydroxyl-coenzyme A-dehydrogenase
BMI = body mass index
C = carbon
CS = citrate synthase
CPT = carnitine palmitoyltransferase
CVD = cardiovascular disease
DEXA = dual energy x-ray absorptiometry
ETC = electron transport chain
EX-BR = exercise before breakfast
FE = fasted exercise (breakfast omission)
FGF21 = fibroblast growth factor 21
GNG = gluconeogenesis
GLP-1 glucagon-like peptide-1
GLUT4 = glucose transport-4 proteins
HDL = high density lipoprotein (**LDL** = low density lipoprotein)
H = hydrogen
HGP = hepatic glucose production
HR = heart rate
IFAB-P = intestinal fatty acid binding protein
mRNA = messenger ribonucleic acid
N = nitrogen
NEFA = non-esterified fatty acids
O = oxygen
PL = phospholipid
PPO = peak power output
RER = respiratory exchange ratio
RPE = rating of perceived exertion
TCA cycle = tricarboxylic acid cycle
T2D = type 2 diabetes
TAG = triglyceride
TCA = tricarboxylic acid
$\dot{V}O_2$ = oxygen uptake (**$\dot{V}O_2$ peak** = peak oxygen uptake)
$\dot{V}CO_2$ = carbon dioxide production
W = watts

CHAPTER 1 - LITERATURE REVIEW

OBESITY AND METABOLIC DISEASE

Obesity can be defined as the excessive expansion of primarily white adipose tissue depots, which can be largely attributed to a long-term energy imbalance, where daily energy intake exceeds the energy requirements of an individual (18). Worldwide, it is estimated that ~ 2 billion adults can be classified as overweight (with a body mass index (BMI) of between 25 and 29.9 $kg \cdot m^{-2}$) and 600 million adults classified as obese, with a BMI > 30 $kg \cdot m^{-2}$ (2). In the UK, the number of adults classified as obese increased from 23 % to 29 % between 2000 and 2016, with the prevalence in 11-15 year olds also rising from 19 % to 23 % during that period (1). These data are reinforced by reports that 39 % of adults in the UK can be classified as having a 'very high' waist circumference [i.e. > 102 cm for men and > 88 cm for women (1)]. The prevalence of obesity in young people is worrying, as it will likely result in a greater number of adults living with the comorbidities of obesity for the majority of their lives, reducing their quality of life and overall life expectancy. Indeed, the Global BMI Mortality Collaboration estimated that the risk of all-cause mortality is 45 % higher in people with class I obesity (BMI: 30.0 to 34.9 $kg \cdot m^{-2}$) and 176 % higher in people with class III obesity (BMI: 40.0 to 59.9 $kg \cdot m^{-2}$), relative to people with a BMI in the healthy range (19).

Although obesity is reported to have directly accounted for < 1 % of deaths from 1995 to 2006 (20), it is associated with a clustering of metabolic diseases, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) and these increase mortality risk (3). In line with the obesity trends, the number of people diagnosed with T2D in the UK has risen > 4 fold since 1980 (21). It is also estimated that in the UK, there will be a further 11 million cases of obesity, 600,000 cases of T2D and 400,000 cases of coronary heart disease and stroke by 2030 (22). Another concern is that the average health care expenditure in the UK is estimated to be 10 %, 23 %, 45 % and 81 % higher for individuals classified as overweight, or with class I, II or III obesity respectively, relative to people with a BMI in the healthy range (23). Indeed, the NHS spends

~ £6 billion in direct medical costs for obesity (> 5 % of their yearly budget) and obesity also incurs productivity losses of ~ £3.3 billion, costs due to absenteeism of ~ £1.3 billion and costs associated with treating metabolic diseases associated with obesity, for example ~ £14 billion for T2D (24). As such, the overall costs of obesity to the UK economy are estimated to be in the region of £50 billion per year or ~ 3 % of gross domestic product and worldwide obesity is estimated to cost ~ £1.32 trillion per year (4). As such, interventions that can successfully reduce obesity prevalence at a population level and/or prevent the development of the associated metabolic diseases are still urgently required. Indeed, reducing obesity and metabolic disease prevalence was one of the primary targets outlined in the '*Global Action Plan for the Prevention and Control of Non-Communicable Diseases: 2013-2020'*, that was produced by the World Health Organisation (25).

FASTING AND POSTPRANDIAL METABOLISM

Metabolic health is an umbrella term that has been used to describe the ability of the body to maintain homeostasis of substrates from a whole-body to a molecular level in response to physiological challenges such as exercise or nutrient intake. The consequences of a sustained positive energy balance (i.e. adiposity) and the individual components of human behaviour that are primarily responsible for the development of obesity (excessive energy intake and/or a sedentary lifestyle) are independent risk factors for many chronic diseases such as T2D and CVD, in part by impairing normal fasting and postprandial substrate metabolism (26-28).

Glucose metabolism

When plasma volume remains unchanged, blood glucose concentrations depend on endogenous and meal-derived rates of glucose appearance *versus* rates of glucose disposal primarily into the liver and peripheral tissues (29). The regulation of blood glucose is primarily coordinated by the actions of the liver, the adipose tissue and skeletal muscle. In a resting overnight fasted state, blood glucose concentrations are maintained at ~ 4-5 $mmol \cdot L^{-1}$ (or ~ 4.6 g for a 70 kg man) in healthy humans (30). This is despite a relatively high rate of blood glucose utilisation, primarily by the brain and to a lesser degree skeletal muscle and the

adipose tissue (31). If the glucose extracted from the blood into peripheral tissues was not replaced, brain and metabolic dysfunction would quickly develop (30). The liver has a key role in regulating blood glucose levels during fasting and extracted blood glucose is largely replaced via hepatic glucose production (HGP) from glycogenolysis (breakdown of glycogen to glucose) and gluconeogenesis [GNG; formation of glucose from non-glucose substrates (30)]. Two hormones primarily regulate blood glucose concentrations and these are insulin and glucagon. Glucagon is released from the α-cells in the pancreas when systemic glucose concentrations are low and upon reaching the liver, glucagon promotes glycogenolysis and inhibits glycogen formation, thus increasing the net secretion of glucose into the systemic circulation (32-34). This response to increased systemic glucagon concentrations helps to prevent hypoglycaemia during fasting. The degradation of glycogen occurs via the coordinated action of glycogen phosphorylase (acting on the terminal α-1, 4-glycosidic linked glucose residues of glycogen) and glycogen debranching enzyme (acting on α-1, 6-branchpoints) to form glucose and glucose-1-phosphate, which can then be converted to glucose-6-phosphate by hexokinase (HK) or phosphoglucomutase, respectively (35). The glucose-6-phosphate can be used in tissues where the glycogenolysis occurred as a substrate for glycolysis (35) or in gluconeogenic tissues (mainly the liver but also the kidneys and intestines) glucose-6-phosphate can be transported to the endoplasmic reticulum, where it can be dephosphorylated to form glucose for secretion into the systemic circulation (35). The breakdown *versus* synthesis of glycogen is primarily regulated by a hormone-triggered cAMP cascade acting via protein kinase A [PKA; (35)]. The main substrates for GNG are lactate and pyruvate, but glycerol and amino acids such as alanine can contribute (31).

The consumption of food provokes a complex endocrine and paracrine response (31), resulting in a profound shift in hepatic and whole-body metabolism from net glucose production to net storage. The carbohydrate content (75-100 g) of meals typically consumed in developed countries could, theoretically, increase blood glucose concentrations by > 8 fold (31). However, at least in metabolically healthy humans, blood glucose concentrations peak at around 8-10 $mmol \cdot L^{-1}$ after most

meals, which is only a 50-60 % increase above normal fasting concentrations (31). In healthy humans, blood glucose concentrations above typical fasting levels are detectable for up to ~ 2 h after 75-100 g of carbohydrate is ingested, although plasma glucose flux can be increased for longer than this (36).

A primary hormone with respect to *postprandial* blood glucose regulation is insulin (37). An increase in blood glucose concentrations stimulates insulin synthesis in the *β*-cells of the pancreas (38) and its secretion into the systemic circulation (39). As blood glucose concentrations rise after carbohydrate consumption, the *β*-cell glucose concentration also increases and this phosphorylates pancreatic and duodenal homeobox 1 (PDX1), which upregulates insulin gene transcription (38). Insulin messenger ribonucleic acid (mRNA) is then translated as preproinsulin. Cleaving of the N-terminal of preproinsulin upon its insertion into the endoplasmic reticulum forms proinsulin, which consists of an amino-terminal *β*-chain, a carboxyl-terminal α-chain and a C-peptide molecule. Within the endoplasmic reticulum, proinsulin is exposed to endopeptidases such as PC1 and PC2 which cleave C-peptide to generate a mature form of insulin. An increase in glucose concentrations in the pancreatic *β*-cells also increases adenosine triphosphate (ATP) production. As a consequence, ATP-sensitive potassium (K+) channels at the plasma membrane close, which disrupts the resting membrane potential and opens the voltage-sensitive calcium (Ca^{2+}) channels. An influx of Ca^{2+} ions into the *β*-cell triggers the exocytosis of secretory vesicles and thus facilitates insulin secretion into the systemic circulation (39). Gastrointestinal-derived peptides such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are also secreted from intestinal enteroendocrine cells after meal ingestion and potentiate this glucose-stimulated insulin secretion response (40).

After meals, a proportion of the exogenous glucose is taken up by the liver in the first-pass extraction from the portal vein and uptake from the hepatic artery (41) the kidneys and the intestine (42), but the remaining meal-derived glucose enters the systemic circulation, before being disposed of by the peripheral tissues (43). Insulin acts to lower blood glucose concentrations by suppressing rates of HGP

(44) and by increasing blood glucose disposal. Skeletal muscle accounts for up to 90 % of insulin-stimulated blood glucose disposal during a hyperinsulinaemic-euglycemic clamp (43) compared to < 5% for adipose tissue (45). However, with oral glucose ingestion, the relative contribution of skeletal muscle to whole-body blood glucose disposal is lower than during a hyperinsulinaemic-euglycaemic clamp at ~ 30-40 % of the ingested glucose, which is broadly similar to the uptake from splanchnic tissues, with the remainder accounted for by the brain, kidneys, heart and the adipose tissue (46, 47). Insulin increases glucose disposal into skeletal muscle by stimulating a translocation of the glucose transporter proteins (GLUT4) from specialised intracellular storage compartments to the cell plasma membrane and also by increasing intrinsic GLUT4 activity (**Figure 1.1**).

At rest and without insulin stimulation up to 90 % of the GLUT4 remain within their intracellular vesicles (48). However, if insulin binds to the extracellular α-subunit of the insulin receptor at the cell plasma membrane, tyrosine residues located in the *β*-subunit are auto-phosphorylated (i.e. insulin receptor tyrosine kinase [IRTK] activation) and can be recognised by a phosphotyrosine-binding domain on the insulin receptor substrate (IRS) proteins (49, 50). As a result the tyrosine residues on the IRS proteins [IRS-1 and IRS-2 are the most important in skeletal muscle and adipocytes (50)] are phosphorylated by IRTK and in turn will bind to the p85 regulatory subunit of Class IA phosphatidylinositol-3-kinase [PI3K; (49, 50)]. At the plasma membrane, the p110 catalytic subunit of PI3K then phosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns-(4,5)-P2] resulting in the formation of phosphatidylinositol (3,4,5)-triphosphate [PtdIns-(3,4,5)-P3] (49, 50). Protein kinase B (Akt/PKB) is then recruited to the cell membrane with the higher cellular PtdIns-(3,4,5)-P3 concentration via its pleckstrin homology domain (PH domain). In skeletal muscle and adipose tissue, Akt2 is the isoform of Akt implicated in the subsequent signalling pathway for GLUT4 translocation and exocytosis (51).

The increased intracellular PtdIns-(3,4,5)-P3 levels produced by PI3K also recruits 3-phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates Akt on Thr^{308}. This leads to activation of Akt and further phosphorylation on Ser^{473}

by mammalian target of rapamycin complex 2 (mTOR-C2). Activated Akt in turn phosphorylates many downstream proteins such as the Rab GTPase activating proteins Akt substrate of 160 kDa (AS160/TBC1D4) and TBC1D1 (52, 53). AS160 and TBC1D1 activity promotes the hydrolysis of GTP to GDP by the Rab proteins on GLUT4 storage vesicles, thereby keeping the GLUT4 vesicles inside the cells. However, if AS160 and TBC1D1 are phosphorylated they become deactivated, leading to increased GTP loading of the Rab proteins on the GLUT4 vesicles, which increases GLUT4 trafficking to the plasma membrane (51, 54). The binding of insulin to the insulin receptor can also phosphorylate a protein known as c-Cbl, which exists as a complex with catabolite activator protein (CAP) (49). There is evidence that with insulin stimulation, this complex is recruited to the plasma membrane and stimulates GLUT4 translocation in the adipose tissue through this pathway, independently of insulin-stimulated PI3K activation (49). Specifically, tyrosine phosphorylated c-Cbl recruits a complex of an adaptor protein - CrkII - and C3G (which is a guanine-nucleotide exchange factor for the GTPase TC10), which then activates TC10 and thereby stimulates GLUT4 translocation (49, 55).

After glucose is transported into the myocyte through the GLUT4 transporter, it is irreversibly phosphorylated by hexokinase (HK) before being stored as glycogen or entering the glycolytic pathway for oxidation (**Figures 1.2 & 1.3**). The HK isoenzymes have a high affinity for glucose but are also inhibited by glucose-6-phosphate, which is the first product formed in the conversion of glucose to pyruvate (32). With increased blood insulin concentrations, skeletal muscle HK (and particularly HK-II) mRNA levels, protein content and activity increases (32). During fasted rest, glucose entering skeletal muscle is directed towards glycogen synthesis *versus* glycolysis in roughly equal proportions and ~ 90 % of glucose entering the glycolytic pathway is oxidised (aerobic glycolysis) with the remainder converted to lactate (anaerobic glycolysis). However, with higher blood insulin levels (after carbohydrate ingestion) glycogen synthase is increasingly activated and glycogen synthesis accounts for > 70 % of glucose disposal into muscle (56).

Insulin activates glycogen synthase through PI3K signalling, by phosphorylating site 1 on a subunit of protein phosphatase 1 (PP1), in a reaction that is catalysed by insulin-stimulated protein kinase-1 (57). Phosphorylation of site 2 on PP1 by PKA inactivates the protein and insulin also decreases PKA activity by activating phosphodiesterase (PDE3B), which converts cyclic adenosine monophosphate (cAMP) to adenosine monophosphate (AMP) (58). Insulin may also increase glycogen synthase activity by inhibiting glycogen synthase kinase-3 (57). Finally, insulin promotes glucose uptake via increased GLUT4 trafficking (**Figure 1.1**) and the consequent increase in glucose 6-phosphate production allosterically activates glycogen synthase (57). Some rodent models suggest that inhibition of glycogen synthase kinase-3 is not rate-limiting for muscle glycogen synthesis and that allosteric regulation of glycogen synthase activity by glucose-6-phopshate may play the more important role in insulin-stimulated muscle glycogen synthesis (59). Therefore, to summarise, insulin promotes net glucose storage as glycogen in skeletal muscle by (*a*) increasing intracellular GLUT4 trafficking (*b*) increasing HK activity/glucose phosphorylation and (*c*) stimulating glycogen synthesis (32).

Insulin can also influence blood glucose concentrations by decreasing hepatic glucose production (HGP). Since it is secreted in the portal vein, the liver is the first tissue encountered by endogenous insulin and HGP can be lowered by a suppression of glycogenolysis via signalling pathways that are activated with the binding of insulin to its hepatic receptor (60). Hepatic insulin action begins with insulin receptor tyrosine kinase (IRTK) activation which, as described previously, leads to PI3K and PDK1/mTORC2 activation to phosphorylate Akt, which in turn, regulates aspects of hepatic glucose and lipid metabolism. Glycogenolysis starts with a formation of glucose-1-phosphate from glycogen, which is catalysed by the phosphorylated ‘a’ active form of glycogen phosphorylase. The inactive ‘b’ form of glycogen phosphorylase is converted to the ‘a’ form by PKA (32). Insulin can supress glycogenolysis by activating PP1 (which converts the ‘a’ to the ‘b’ form of glycogen phosphorylase) and by inhibiting PKA. However, insulin infused peripherally can also be as effective for suppressing HGP as intra-portal insulin infusions, suggesting a likely role for peripheral tissues in the suppression of HGP

(61). Glucagon stimulates HGP but systemic glucagon concentrations decrease with insulin infusions (62), because insulin inhibits glucagon secretion from the pancreatic α-cells (63). This effect of insulin may contribute to a suppression of HGP, because when blood glucagon concentrations are maintained during an insulin infusion, the suppression of HGP is lessened (64). Insulin also decreases the mobilisation of non-esterified fatty acids (NEFA) and glycerol from the adipose tissue and amino acids from skeletal muscle, reducing their availability for GNG in the liver (61). Insulin-mediated changes in adipokine secretion (61) and at least in rodents, a signalling pathway from the brain to liver may also help to regulate a suppression of HGP with higher blood insulin concentrations (65). Therefore, direct hepatic signalling and indirect (i.e. peripheral) effects likely contribute to an insulin-induced inhibition of HGP following nutrient ingestion.

Fatty acid metabolism

The amount of energy stored in the human body as glycogen is limited at around 100 g in the liver and 350-700 g in skeletal muscle, with smaller depots in the brain, heart, kidneys and the adipose tissue (66). This limited storage capacity is mainly due to the low energy density of carbohydrates and the implications this would have for the mass that would need to be moved during physical activity. Humans can also utilise lipids as a fuel for energy metabolism and compared to glucose, fatty acids have a higher energy density [~ 40 $kJ \cdot g^{-1}$ for palmoyl-stearol-oleoyl glycerol [PSOG] which is representative of triglyceride [TAG] from adipose tissue relative to ~ 15 $kJ \cdot g^{-1}$ for glucose (31, 67)]. As such, the capacity of the human body for storing lipids is greater than carbohydrates at ~ 10 kg for a lean 75-80 kg man (66). In humans, lipids are mostly stored as TAG, which are formed from 3 fatty acids and 1 glycerol molecule, primarily in the subcutaneous adipose tissue, although skeletal muscle (~ 300 g for a lean 75-80 kg man) and the liver also have their own smaller, stores (31). The adipose tissue is mostly comprised of adipocytes, which exist in a matrix of blood vessels, immune cells, collagen and fibroblasts. A delivery of fatty acids to the adipose-tissue is mainly from chylomicrons derived from the intestine after the ingestion of a meal, and very low-density lipoproteins (VLDL) derived from the liver. The glycerol base used for

the synthesis of TAG can be obtained either from glycerol-3-phosphate which is derived from glycolysis, or from oxaloacetate via glycerolneogenesis (68).

Lipids are insoluble in aqueous solutions, but are soluble in organic solvents and therefore in order to be transported in the plasma, the majority of lipids are bound to albumin for non-esterified fatty acids (NEFA) or carried in lipoprotein particles. Depending upon the methodology used and participants studied, plasma NEFA concentrations range between ~ 300-600 $\mu mol \cdot L^{-1}$ in the overnight fasting state, to ~ 1,300 $\mu mol \cdot L^{-1}$ after a 72-h fast (69). Cholesterol is not a metabolic energy source but is an essential component of plasma membranes and a precursor for steroid hormones and a variety of other specialised molecules (32). Cholesterol, TAG and phospholipids (molecules containing 2 fatty acids with a water-soluble phosphate end) are transported in the systemic circulation as complexes called lipoproteins, which are made from lipids and proteins and are classified according to their size and lipid content (32). VLDL and low-density lipoproteins (LDL) are TAG- and cholesterol-rich particles respectively, which are transported from the liver to the peripheral tissues, whereas high-density lipoproteins (HDL) remove excess cholesterol from the periphery and return it to the liver for excretion (32).

When humans ingest a meal containing fat, the dietary fat is digested and broken down into NEFAs and monoglycerides, before being re-esterified in the intestine. Dietary lipids enter the systemic circulation as apolipoprotien B48 (ApoB48)-TAG-rich chylomicrons, which transport lipids from the intestine to the peripheral tissues for utilisation or storage (32). Blood TAG concentrations typically increase steadily after the ingestion of a meal containing fat and peak at ~ 5 h post-meal, whereas (especially if the meal elicits an insulin response), NEFA concentrations are decreased, often to negligible levels (70, 71). The ingestion of 20-30 g of dietary fat causes smaller perturbations to systemic TAG concentrations (often not > 1.0 $mmol \cdot L^{-1}$) relative to the changes in blood glucose concentrations that can occur after a carbohydrate-rich meal is ingested (31). In skeletal muscle and the adipose tissue, TAG derived from chylomicrons, VLDL and LDL is hydrolysed by lipoprotein lipase (LPL) to its fatty acid and glycerol sub-components, before

TAG resynthesis can then occur (32). If a mixed-macronutrient meal is consumed by healthy humans, insulin also plays a role in dietary fat trafficking, diverting TAG towards storage in the adipose tissue by up-regulating LPL activity in the adipose tissue, down-regulating LPL activity in muscle and supressing the mobilisation of adipose-tissue derived fatty acids and liver-derived lipid [as discussed later in this literature review (32)]. In humans, the primary depot from where fatty acids are liberated for energy metabolism is the subcutaneous adipose tissue, due to the larger size of this store compared to visceral adipose tissue (72).

The lipolysis of TAG occurs in 3 main stages (**Figure 1.4**). The first hydrolysis is catalysed by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) and this forms diacylglycerol (DAG) and releases a non-esterified fatty acid chain (NEFA). DAG can be further hydrolysed by HSL to release a second NEFA molecule and a third NEFA can be released with a hydrolysis of monoacylglycerol (MAG) by monoacylglycerol lipase (MAGL). Adipose-tissue derived NEFA can be transported to skeletal muscle for metabolism, where they undergo *β*-oxidation, before the tricarboxylic acid cycle (TCA) and the electron transport chain (ETC) occur (**Figures 1.5 & 1.6**). Alternatively they can undergo re-esterification and remain in the adipocyte (73) and during prolonged fasting > 40 % of NEFA can be recycled back into TAG in this manner (74). However, as glycerol kinase is not present in human adipose tissue, glycerol is still released into the systemic circulation despite fatty acid re-esterification. As such, the rate of appearance of glycerol into the systemic circulation has been used to assess rates of lipolysis.

During fasting, *β*-adrenergic receptor activation in the adipose-tissue stimulates adenylyl cyclase activity and the increased production of cAMP (75). The cAMP molecule binds to and activates PKA, which in turn, increases the activation of proteins that regulate lipolysis, including HSL (75). Perilipins form a protective barrier for TAG against intracellular lipase activity, but with the activation of PKA, a conformational change occurs, which permits greater access to the lipid within the droplet (76). Whilst not a main focus of this review, other hormones stimulate lipolysis, such as growth hormone, tumour necrosis factor alpha (TNF-α), cortisol

(58) and interleukin 6 (IL-6) (77). AMPK (5′- adenosine monophosphate activated protein kinase) is also activated under conditions of lower energy availability and promotes catabolic pathways in part due to its inhibitory effects on acetyl-CoA carboxylase (ACC); the enzyme responsible for catalysing the carboxylation of acetyl coenzyme A (acetyl-CoA) to malonyl coenzyme A (malonyl-CoA), in the first committed step of lipogenesis (78). Thus, in healthy humans the contribution of NEFA to metabolism is higher than glucose in a fasted, rested state (31, 79).

However, following the ingestion of a carbohydrate-rich meal, higher blood insulin concentrations reduce the net mobilisation of adipose-derived fatty acids (80). In healthy humans, the EC_{50} of insulin (the concentration to facilitate a half-maximal response) to supress lipolysis is ~ 50 % of the EC_{50} of insulin for supressing HGP, showing the sensitivity of adipose tissue to insulin (81). As stated previously, the binding of insulin to the insulin receptor results in the tyrosine phosphorylation of IRS proteins and binding of PI3K. The consequent activation of PI3K inhibits PKA activity via Akt signalling (**Figure 1.4**). Insulin also increases net glucose disposal into the adipose tissue by increasing GLUT4 trafficking (via Akt signalling) and in the presence of insulin, hydrolysed NEFA are increasingly re-esterified, using glycerol-3-phosphate from glucose entering the adipocyte to re-form TAG (31). Insulin can also stimulate enzymes involved in the esterification of fatty acids to glycerol, including glycerol-3-phosphate acyltransferase, which catalyses the rate limiting step in TAG synthesis in adipose tissue (82). This effect of insulin partly occurs via sterol regulatory element binding proteins, such as SREPB-1c, which activate the transcription of genes encoding lipogenic enzymes (83). In contrast, in the absence of insulin, lipogenic pathways are suppressed and ATGL and HSL (which are inhibited by insulin via PKA signalling), liberate fatty acids from their glycerol base, allowing them to be secreted into the systemic circulation.

The glucose-fatty acid cycle (Randle cycle)

In a transition from fasting to feeding, insulin stimulates increased blood glucose disposal and carbohydrate utilisation by skeletal muscle and supresses rates of lipolysis in the adipose tissue, which reduces the availability of adipose-derived

NEFA for metabolism (84). In addition, higher rates of lipid availability reduces glucose utilisation in skeletal muscle. When TAG are infused intravenously and in concert with heparin (which activates LPL, thus allowing it to interact with the infused TAG to release NEFA) peripheral blood glucose disposal is decreased (85). This regulation of metabolism according to systemic substrate availability and the co-ordination between the liver, skeletal muscle and the adipose tissue in this regard, reduces competition for substrate utilisation in skeletal muscle (84).

A regulation of metabolism according to substrate availability also occurs *within* skeletal muscle. NEFA taken up into skeletal muscle are first transported across the mitochondrial membrane before they undergo *β*-oxidation, to form acetyl-CoA for the TCA cycle (31). An accumulation of acetyl-CoA, NADH and citrate can supress pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK) activity respectively (86) and these enzymes have roles in pathways that convert glucose to pyruvate and the decarboxylation of pyruvate to acetyl CoA (**Figure 1.2**). This results in glucose-6-phosphate accumulation, an inhibition of HK and ultimately a reduction in glycolysis (86). In turn, an increased utilisation of glucose increases malonyl-CoA production, which inhibits the carnitine palmitoyltransferase (CPT) mediated transport of lipids across the mitochondrial membrane (86). An ability to adjust metabolism to substrate availability and energy demands is a key aspect of metabolic health (87). For example, in response to eating a carbohydrate-rich meal, metabolic flexibility represents the efficient transition in substrate utilisation in favour of carbohydrates from mostly lipids during fasting. The purpose of this response is to move from a catabolic state where glycogenolysis and lipolysis meet energy demands during fasting, to a more anabolic state where the ingested energy is utilised or stored in the liver, adipose tissue or skeletal muscle (87).

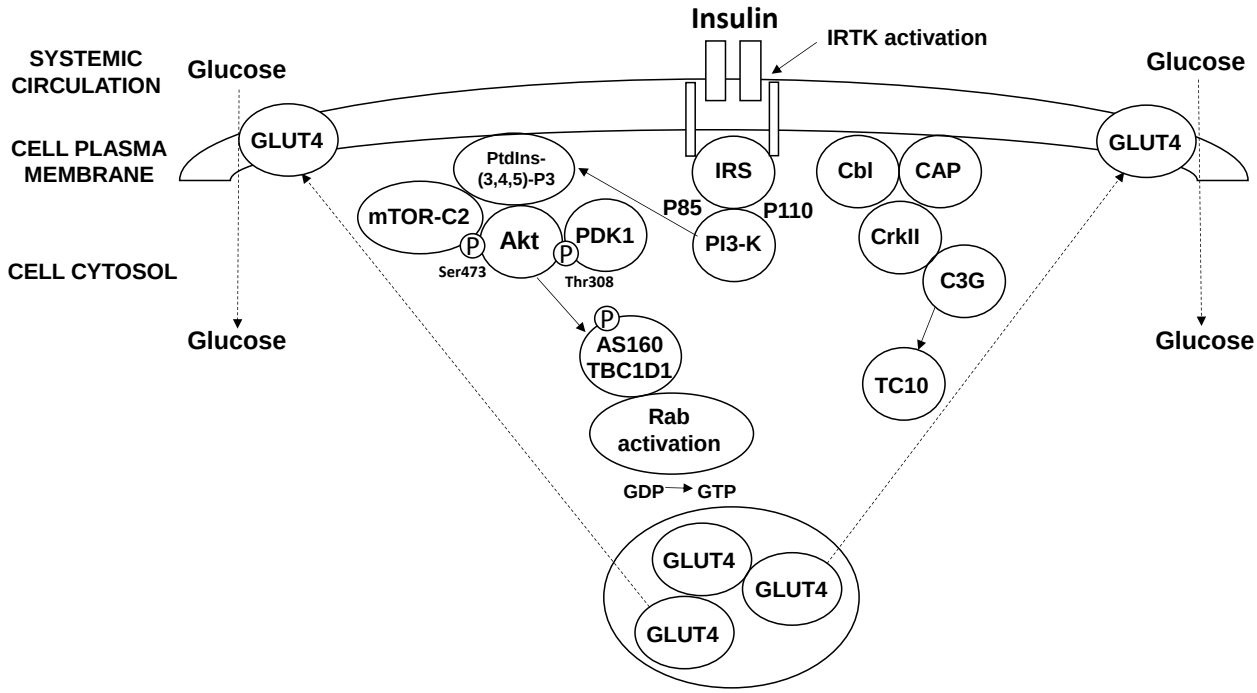


Figure 1.1. Insulin-stimulated GLUT4 translocation in skeletal muscle and the adipose tissue. Insulin binding to the insulin receptor at the plasma membrane results in the phosphorylation (and activation) of Akt. In turn, this results in the phosphorylation (and deactivation) of proteins downstream of Akt such as AS160 and TBC1D1, which regulate the processes involved in GLUT4 translocation from their intracellular vesicles to the plasma membrane. The activation of a Cbl-CAP complex is also thought to mediate insulin-stimulated GLUT4 trafficking in the adipose tissue. Drawn using information provided in (49, 50).

Abbreviations:

GLUT4 = glucose transporter 4
IRTK = insulin receptor tyrosine kinase
IRS = insulin receptor substrate
PI3-K = phosphoinositide 3-kinase
PtdIns-(3,4,5)-P3 = phosphatidylinositol (3,4,5)-trisphosphate
PDK1 = 3-phosphoinositide-dependent kinase-1
mTOR-C2 = mammalian target of rapamycin complex 2
Akt = protein kinase B
AS160 = Akt substrate of 160 kDa
CAP = Cbl-associated protein
C3G = RapGEF1

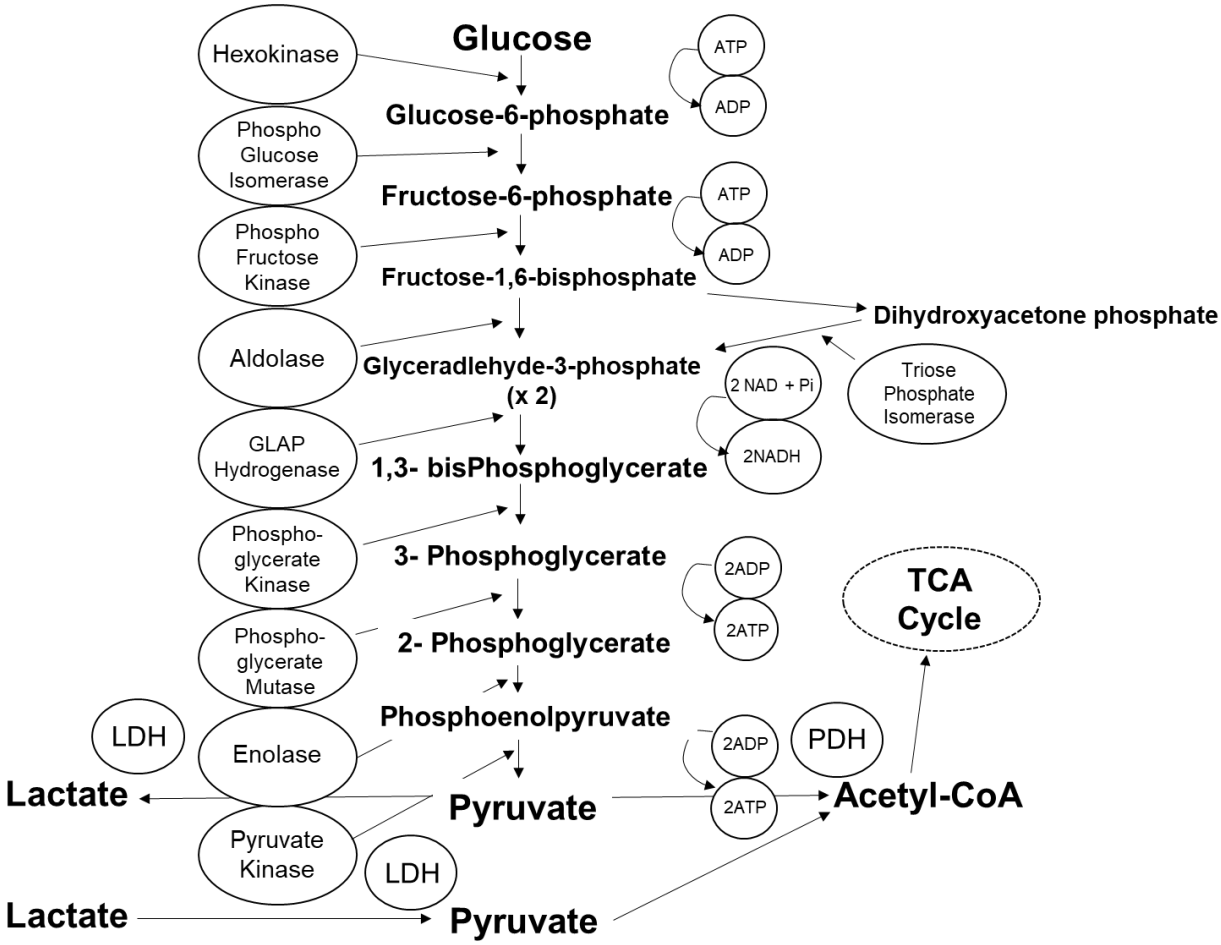


Figure 1.2. The formation of Acetyl-CoA from glucose (i.e. glycolysis) for the tricarboxylic acid cycle (TCA cycle). As fructose 1,6, bisphosphate is unstable, it splits into two 3 carbon products, so two 3 carbon (Pyruvate) molecules are produced for each glucose molecule. Drawn using information provided in (32).

Abbreviations:

TCA cycle = tricarboxylic acid cycle or the Krebs cycle (see **Figure 1.6**)
LDH = Lactate dehydrogenase
PDH = Pyruvate dehydrogenase
NAD+/NADH = nicotinamide adenine dinucleotide (oxidised/reduced form)
ATP = adenosine triphosphate
ADP = adenosine diphosphate

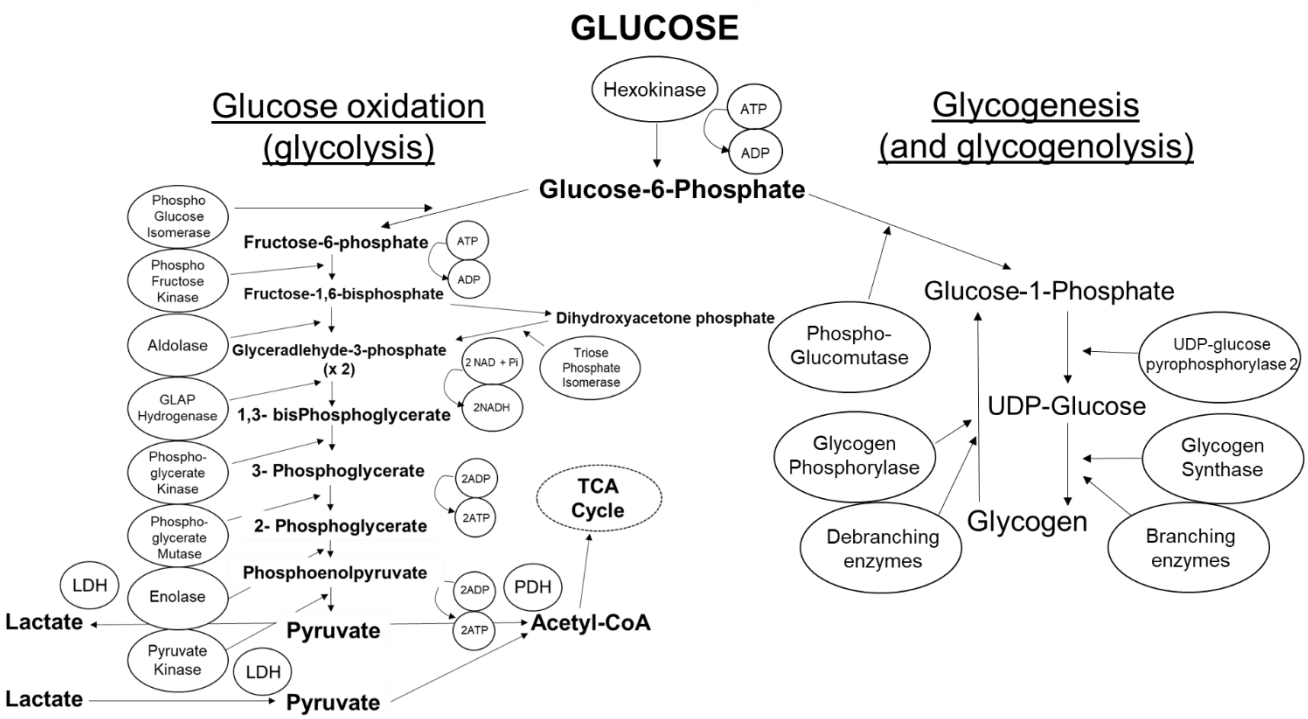


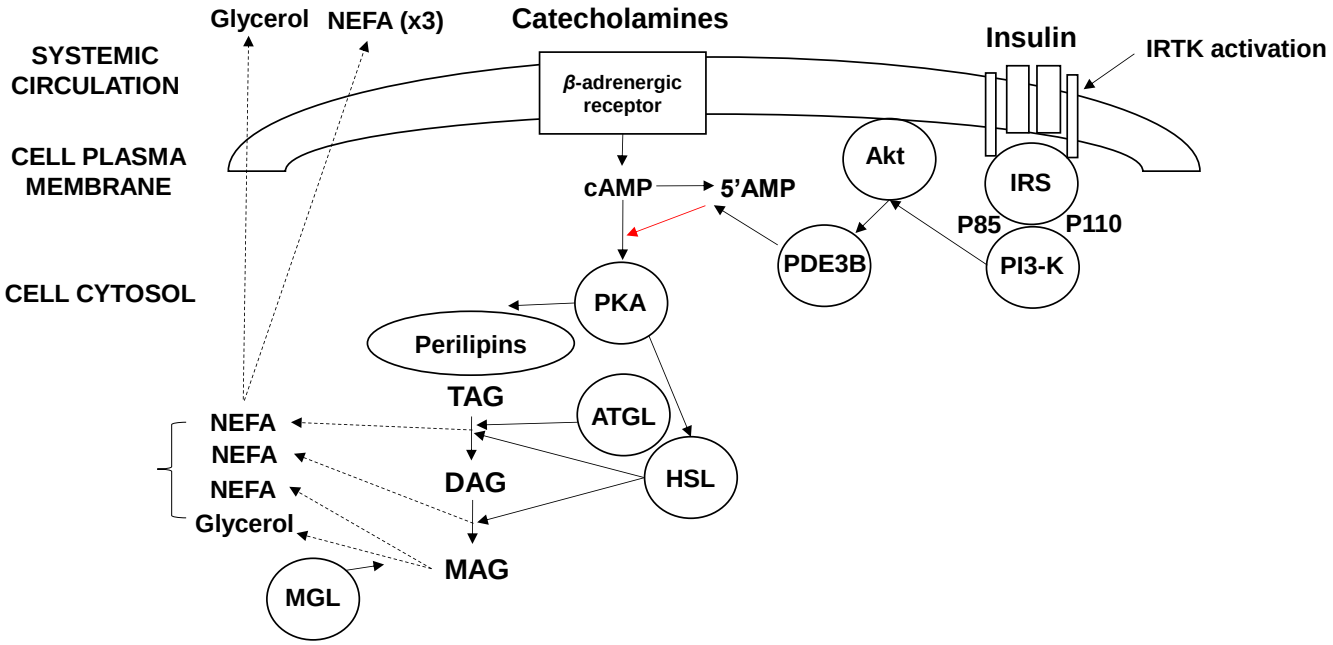
Figure 1.3. Pathways by which glucose can be oxidised (glycolysis) or converted to glycogen (glycogenesis) after it has been transported into skeletal muscle and phosphorylated by hexokinase. Drawn using information provided in (32).

Abbreviations:

TCA cycle = tricarboxylic acid cycle or the Krebs cycle (**Figure 1.5**)

LDH = Lactate dehydrogenase

PDH = Pyruvate dehydrogenase



637
638 **Figure 1.4.** The lipolysis of triglycerides (TAG). Catecholamines stimulate- and
639 insulin inhibits lipolysis, primarily acting via pathways which regulate the activity
640 of protein kinase A (PKA). In this figure the red arrow denotes an inhibitory effect
641 of Akt signalling (decreasing cAMP concentrations) on PKA activity. Drawn using
642 information provided in (75).
643
644 **Abbreviations:**
645 IRTK = insulin receptor tyrosine kinase
646 IRS = insulin receptor substrate
647 PI3-K = phosphoinositide 3-kinase
648 Akt = protein kinase B
649 PDE3B = phosphodiesterase 3B
650 PKA = protein kinase A
651 TAG = triglyceride or triacylglycerol
652 DAG = diacylglycerol
653 MAG = monoacylglycerol
654 ATGL = adipose triglyceride lipase
655 HSL = hormone-sensitive lipase
656 MGL = monoacylglycerol lipase
657 NEFA = non-esterified fatty acid
658 cAMP = cyclic adenosine monophosphate

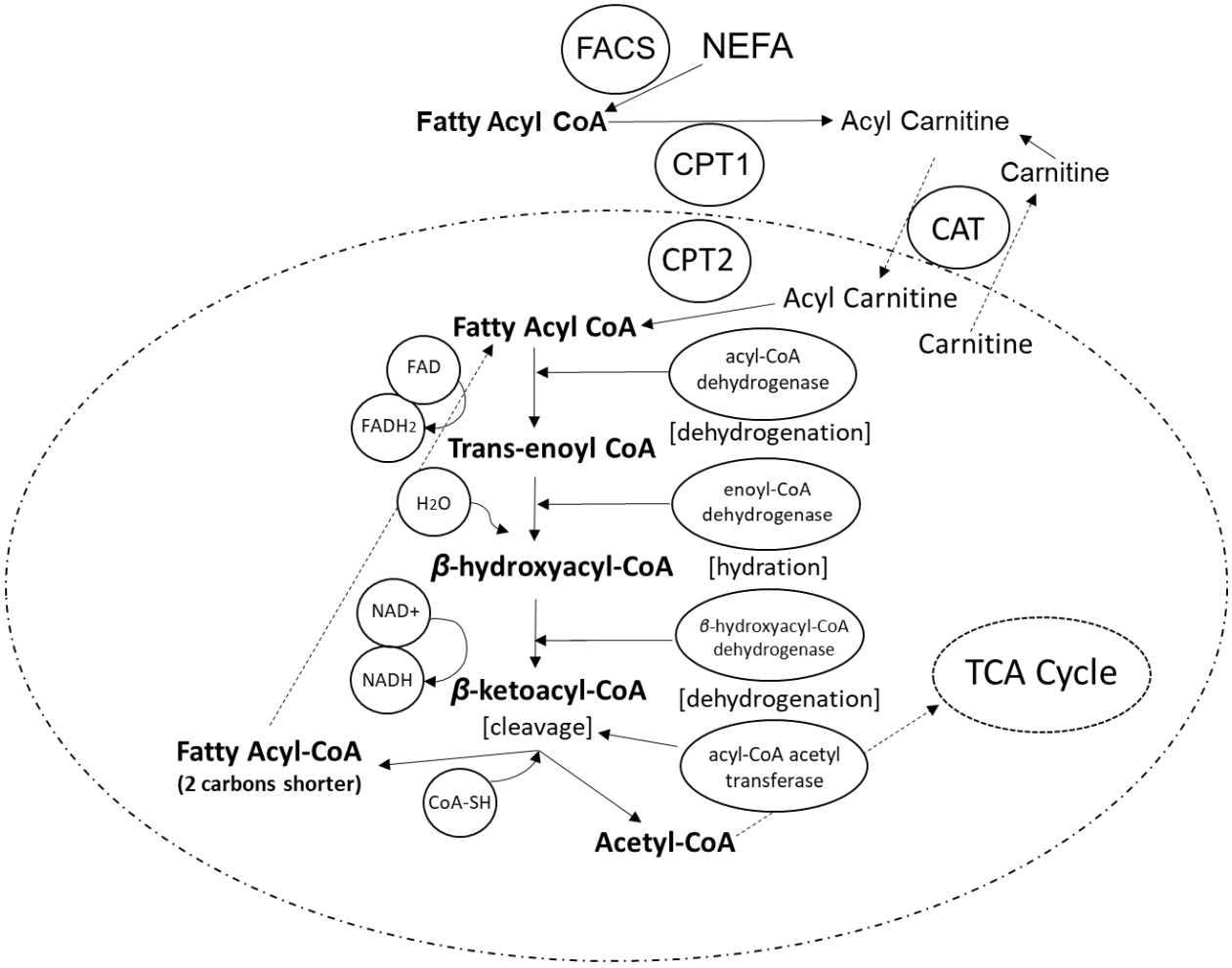


Figure 1.5. The *β*-oxidation of fatty acids. This occurs until the original fatty acyl chain is separated into Acetyl-CoA molecules for the tricarboxylic acid cycle (TCA cycle). Drawn using information provided in (32).

Abbreviations:

CAT = carnitine-acylcarnitine translocase (responsible for transporting carnitine-fatty acid complexes and carnitine across the inner mitochondrial membrane)
FACS = fatty-acyl-CoA synthase
CPT1 = Carnitine palmitoyltransferase I
CPT2 = Carnitine palmitoyltransferase II
TCA cycle = tricarboxylic acid cycle or the Krebs cycle (**Figure 1.6**)

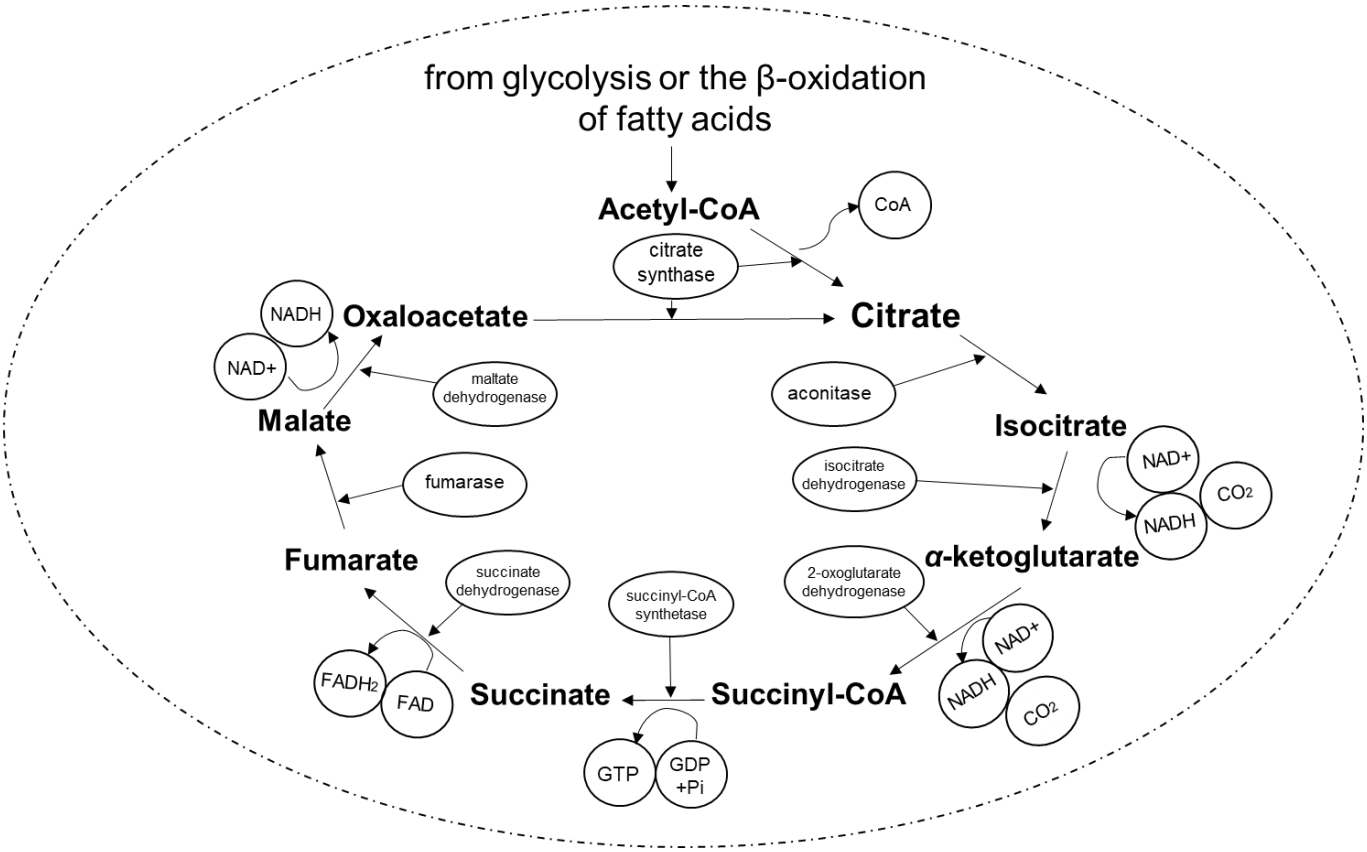


Figure 1.6. The tricarboxylic acid cycle (TCA cycle). This is a series of reactions that utilises the Acetyl-CoA molecules that have been derived from carbohydrates (**Figure 1.2**) and lipids (**Figure 1.5**) to form substrates for the electron transport chain (and the production of ATP). Drawn using information provided in (32).

Abbreviations:

NAD+/NADH = nicotinamide adenine dinucleotide (oxidised/reduced form)

FAD/$FADH_2$ = flavin adenine dinucleotide (oxidised/reduced form)

CO_2 = carbon dioxide

GTP = guanosine-5'-triphosphate

GDP = guanosine-5'-diphosphate

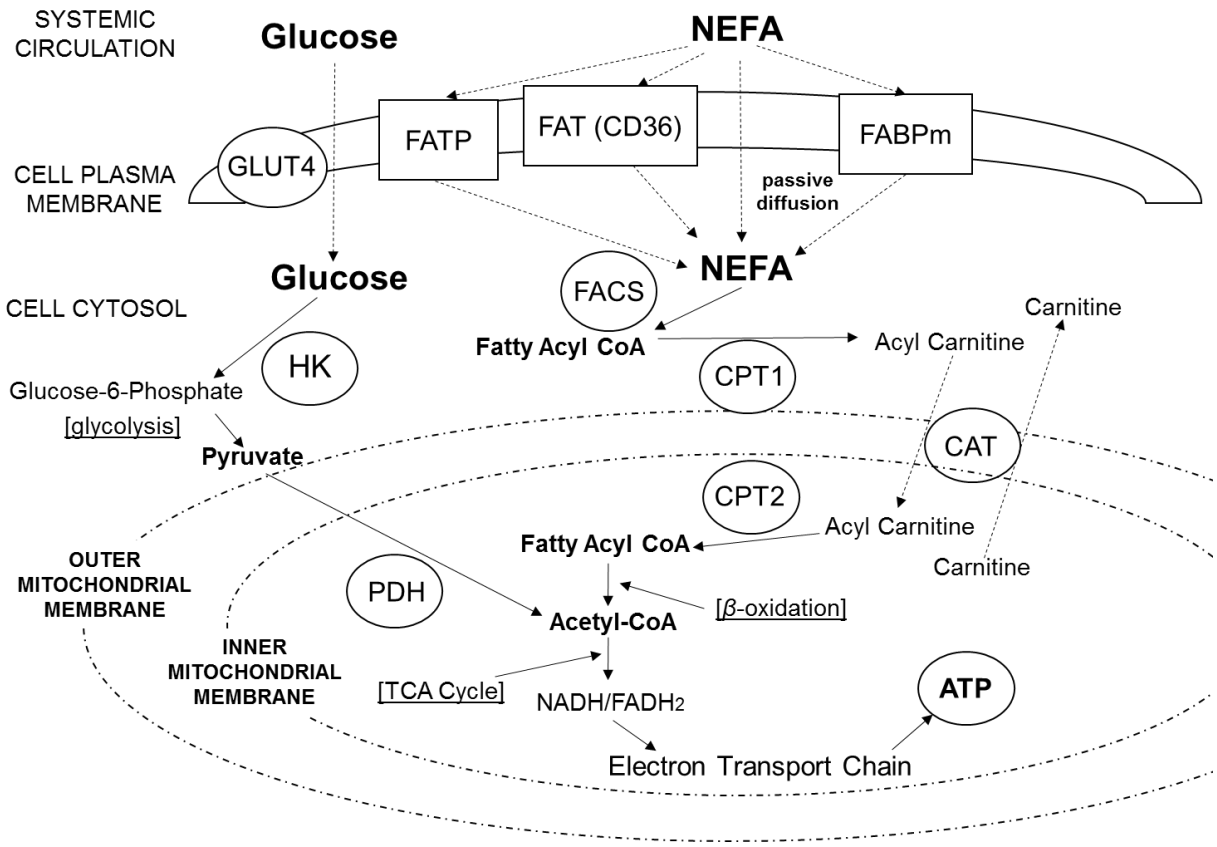


Figure 1.7. NEFA transport within muscle cells (for glucose see **Figure 1.1**). Various transporter proteins (and passive diffusion) facilitate NEFA transport across the cell plasma membrane. Transport across the mitochondrial membrane occurs via the carnitine palmitoyltransferase (CPT) system. Also see **Figure 1.5** for the *β*-oxidation of fatty acids and **Figure 1.6** for the TCA cycle. Drawn using information provided in (32, 84).

Abbreviations:

GLUT4 = glucose transporter 4
FATP = fatty acid transport protein
FAT/CD36 = fatty acid translocase/cluster of differentiation 36
FABPm = fatty acid binding protein
FACS = fatty-acyl-CoA synthase
HK = hexokinase
PFK = phosphofructokinase-1
PDH = pyruvate dehydrogenase
CPT1 = carnitine palmitoyltransferase I
CPT 2 = carnitine palmitoyltransferase II
CAT = carnitine-acylcarnitine translocase
TCA cycle = tricarboxylic acid cycle (or the Krebs cycle)
NEFA = non-esterified fatty acid
ATP = adenosine triphosphate

METABOLIC FLEXIBILITY AND INSULIN SENSITIVITY

Impaired fasting metabolism

The respiratory exchange ratio (RER) can be used to assess rates of substrate oxidation (88). The RER is an indirect measure of the respiratory quotient (RQ), which is the quantity of carbon dioxide (CO_2) produced relative to oxygen (O_2) consumed by a given tissue at any specific time (88). At the whole-body level, the ratio of CO_2 produced to O_2 consumed depends on the ratio of substrates being oxidised (88). The first reported limb balance study was in 1956, with measures of glucose and lipid utilisation via the RQ of the forearm muscle in healthy humans (89). This showed the transition in substrate utilisation in skeletal muscle from mainly lipids during fasting, towards carbohydrates after feeding. However, it has since been shown that the resting, fasted RQ of skeletal muscle can be higher in humans with obesity and T2D *versus* healthy humans (90, 91). This has also been shown at a whole body level using the RER (92).

These results therefore suggests that at least some people with obesity display abnormalities in the ability to use lipids as a main substrate for energy metabolism in a resting, fasted state. This may be due to a reduced mitochondrial oxidative capacity (~ 20-40 %) in humans with *versus* without obesity (90, 93) and/or an increased presence of hyperinsulinemia and consequent suppression of lipolysis in the adipose tissue (as discussed later in this review). However, a difference in resting, fasting substrate utilisation in humans with *versus* without obesity is not always shown (94). Skeletal muscle has a capacity to increase O_2 uptake per kg of tissue by ~ 150-fold during exercise (95) and at the whole body level, O_2 uptake can increase by > 10 fold in the transition from rest to exercise (96). Thus, the relatively small decrease in mitochondrial oxidative capacity (~ 30 %) shown in some humans with obesity may be insufficient to impair lipid metabolism during resting conditions [with any inter-individual variability also potentially attributable to differences in their cardiorespiratory fitness levels (97)]. However, obesity is also associated with other impairments in energy metabolism, including in the ability to switch from predominantly lipid utilisation during periods of fasting to carbohydrate utilisation after carbohydrate consumption (90, 91).

Impaired postprandial metabolism

As early as 1931 it was proposed that insulin resistance - the reduced action of insulin - was instrumental in the onset and development of T2D (98). It has since been proposed that increased peripheral insulin resistance may also explain any link between the metabolic risk factors that characterise obesity, T2D and CVD (99). In skeletal muscle, insulin resistance primarily manifests as impairments in insulin-stimulated blood glucose disposal (99). Studies using hyperinsulinaemic-euglycemic clamps have shown that obesity and T2D are associated with ~ 50 % lower rates of insulin stimulated blood glucose disposal *versus* healthy humans (100) and this has been mainly attributable to skeletal muscle (43). Humans with obesity but with normal glucose tolerance also show increased insulin resistance in skeletal muscle compared to lean, age- and sex-matched controls and the severity of this impairment has also been correlated with BMI (101, 102).

A lower rate of insulin-stimulated peripheral glucose disposal in obesity has been attributable to defects that impair glucose transport, glucose phosphorylation and glycogen synthesis in skeletal muscle (100). However, muscle from humans with obesity or T2D does not show a reduced *GLUT4* mRNA expression or GLUT4 protein content compared to muscle from healthy humans, suggesting that the transcriptional or translational regulation of GLUT4 is not primarily responsible for this impairment (103). Instead, reduced insulin-stimulated GLUT4 *translocation* (104) and impaired HK and glycogen synthase activation (105) are more likely to be responsible. Some studies show a reduction in insulin binding to myocytes from humans with obesity or T2D (106). However, a reduction in insulin receptor number is not present in many insulin-resistant humans and does not correlate with the severity of insulin resistance (107). As such, impaired intramuscular insulin signalling may not be primarily related to defects in insulin binding (100). Some (106) but not all (108) studies report an impaired phosphorylation of the tyrosine residues on IRS proteins in T2D that is not explained by insulin receptor number or insulin binding affinity. Others have also shown that the severity of the defect (relating to impaired P13K activation) can be correlated with the decrease in insulin-stimulated blood glucose disposal rates and glycogen synthase activity

(109). An increased serine phosphorylation of IRS-1 in insulin resistant muscle may impair the tyrosine phosphorylation of IRS-1, but the factor(s) responsible for this effect in obesity is currently unclear (100). An increased activity of protein kinase C (PKC) is also present in insulin resistant muscle and may have a role in disrupting insulin signalling (100), as discussed subsequently.

Whilst this review has a primary focus on skeletal muscle as the primary site of postprandial blood glucose disposal, insulin resistance can also occur in other tissues with obesity. In the liver, insulin resistance impairs (insulin-stimulated) glycogen synthesis and the suppression of HGP (110). Gene knock-out models suggest that in rodents this is due to increased hepatic DAG concentrations which activates PKC and impairs IRTK activation (111, 112). In humans, the hepatic DAG content and heightened PKC activation (but less so the ceramide content), can also predict hepatic insulin resistance (113). Hepatic steatosis can however develop without detectable impairments in hepatic insulin sensitivity (111). In some instances, hepatic TAG synthesis may be driven by increased fatty acid or carbohydrate (especially fructose) delivery (111). This can manifest as increased TAG synthesis and storage in the liver (as reported in non-alcoholic fatty liver disease), which can then worsen insulin resistance (111). In the adipose tissue, insulin resistance impairs (insulin-stimulated) glucose and fatty acid disposal and the suppression of lipolysis (99). Although insulin-stimulated glucose disposal in adipose-tissue is minor relative to skeletal muscle (~ 5 % of whole-body glucose disposal), glucose metabolism in adipocytes may alter fatty acid secretion via transcription factors including carbohydrate response element binding protein, which co-ordinates the transcriptional regulation of enzymes that traffic glycolytic end-products towards lipogenesis (111). Finally, around 120 $pmol \cdot L^{-1}$ of insulin results in a half-maximal suppression of lipolysis in healthy adipocytes but around 300 $pmol \cdot L^{-1}$ is needed for the same effect in insulin-resistant adipose tissue (114). In insulin-resistant adipose tissue, a higher rate of fatty acid secretion then increases the likelihood of lipid synthesis occurring in the liver (111). Defective glucose disposal in skeletal muscle also increases glucose availability to the liver, providing another substrate for *de novo* lipogenesis and TAG synthesis (111).

Therefore, peripheral insulin resistance across multiple tissues results in fasting and postprandial hyperglycaemia (due to lower blood glucose disposal rates and impairments in the suppression of HGP) and hyperlipidaemia (due to an impaired suppression of lipolysis in adipose tissue but maintenance of fatty acid synthesis in the liver) (99). Studies in rodents suggest that hepatic insulin resistance may precede insulin resistance in other tissues (115). Nonetheless, in humans, insulin resistance in the liver has been suggested to occur concomitantly with insulin resistance in skeletal muscle (116). Despite some uncertainly in that regard, the consequent hyperglycaemia and hyperlipidaemia are important in the continual development of T2D and CVD, despite an initial increase in *β*-cell function (i.e. insulin secretion) initially compensating for defects in insulin action (100, 111).

Lipid metabolism and insulin resistance

With a sustained positive energy balance an expansion of the adipose tissue occurs via an increased number of adipocytes (i.e. hyperplasia) and hypertrophy of existing adipocytes. In particular, visceral adipose mass seems to be important for insulin sensitivity (117). The exact mechanisms underpinning any association between an increased fat mass and insulin resistance are still unclear. However, because energy metabolism is primarily regulated by the liver, adipose tissue and skeletal muscle and because insulin has effects across all of these tissues, it is unlikely that insulin resistance is attributable to one single factor. Nonetheless, since the work of Randle *et al.* (84) a specific role of impairments relating to lipid metabolism in the aetiology of insulin resistance has been investigated. Insulin sensitivity is impaired in myocytes exposed to adipocyte-derived lipids (118), which supports the theory that increasing lipid availability to skeletal muscle may worsen insulin resistance (119). Skeletal muscle sampled from humans with obesity often has a higher lipid content than humans without obesity, which has been demonstrated using muscle biopsy samples (120), histological staining (121), computed tomography (122) and magnetic resonance spectroscopy [MRS; (123)]. In insulin-resistant humans, the lipid content of skeletal muscle can also be correlated with the severity of insulin resistance, even after an adjustment for total visceral fat mass (120). Whilst early studies did not distinguish between

intramyocellular- (IMCL; lipid droplets within myocytes) *versus* extramyocellular-lipid (EMCL; lipid located between myocytes) studies using MRS now suggest that IMCL can be more strongly correlated with peripheral insulin resistance, despite representing a small proportion of the intramuscular lipid pool (124-126). However, people who regularly exercise and often have high levels of insulin sensitivity, also have higher muscle lipid contents than sedentary people matched for body fat (127). Thus, for insulin sensitivity, the intramuscular lipid content should be interpreted in a context of factors such as the mitochondrial oxidative capacity of the muscle and how often intramuscular lipid stores are used (119).

Any negative effect of lipid accumulation on insulin sensitivity (lipotoxicity) occurs when some of the lipid entering the mitochondria is only partly degraded. Lipid species such as DAG, fatty acyl-CoA esters and ceramide can then accumulate and have been proposed to interfere with insulin signalling (128-130), ultimately manifesting as impairments in insulin-stimulated glucose disposal (**Figure 1.8**). Increased intracellular DAG concentrations may increase PKC activity and impair IRS-1 activation (131), with ceramide acting on more distal steps within the insulin signalling cascade by inhibiting Akt2 and glycogen synthase kinase activation (132). However, the evidence linking the lipid intermediates to insulin resistance has mostly been derived from rodent models and in humans a mediating role for DAG or ceramide in the onset of insulin resistance is not always clear (133).

Another factor that may, at least partly, be responsible for a link between obesity, lipid metabolism and insulin resistance is alterations to the phospholipid (PL) content of skeletal muscle (134). Although demonstrating causality is difficult, an increased saturated fatty acid content and decreased polyunsaturated fatty acid [PUFA; and in particular *n*-3 PUFA] content of skeletal muscle has been shown in metabolic disease states, including T2D (135). In addition, when rats are fed a high-fat diet they become insulin resistant, but the inclusion of *n*-3 PUFA in the diet can alleviate insulin resistance, if PUFA derivatives are incorporated into the intramuscular PL pool (136). Cross-sectional research in humans also suggests that the PL content of muscle may have a role for insulin resistance. In one study,

rectus abdominis and fasting blood samples were taken from coronary artery disease patients and *vastus lateralis* samples collected and a hyperinsulinaemic-euglycaemic clamp was performed in healthy men (137). In the patients, fasting insulin concentrations were negatively correlated with the PUFA content of the intramuscular PL pool (independently of age, sex or whole-body adiposity) and in healthy men, the PUFA content of the PL pool was positively correlated with insulin sensitivity. These results were explained by the theory that properties of cell membranes are determined by their PL composition as increasing the PUFA content of cultured cells increases membrane fluidity, insulin receptor number and insulin action (138-140), and this effect is reversed if the saturated fatty acid content of the membranes is increased (140). The PL content of muscle may also alter insulin sensitivity via signalling molecules derived from C20 PUFAs (141).

Whilst this review has a focus on skeletal muscle, it should be acknowledged that the systemic concentration of adipose-tissue derived hormones and inflammatory cytokines that can also impair peripheral insulin sensitivity, increases with the expansion of visceral fat mass in humans with obesity (142). For example, adipocytes from humans with obesity overexpress TNF-α, which is associated with disrupted glucose uptake in skeletal muscle and adipose tissue (143, 144). Further evidence that low-grade inflammation contributes to insulin resistance in obesity is that anti-inflammatory treatments can lower blood glucose concentrations and increase peripheral insulin sensitivity (145, 146). In addition, some inherent characteristics of the adipose-tissue are altered in obesity, for example a reduction in blood flow can occur, which reduces the capacity for nutrient and O_2 delivery (147). This effect is believed to play an important role in the development of a pro-inflammatory state and may also impair the ability of the adipose-tissue to store lipids (and by doing so, increase the likelihood of ectopic storage in the liver and skeletal muscle) (148). Consequently, an association between obesity and insulin resistance is unlikely to be completely attributable to defects in skeletal muscle. However, changes in skeletal muscle are likely to have an important role, and these appear to relate to lipid metabolism.

Hyperinsulinemia via primary insulin hyper-secretion might also occur ahead of peripheral insulin resistance and drive increases in body mass and decreases in insulin sensitivity (149, 150). For example, hyperinsulinemia suppresses lipolysis within the adipose tissue and can inhibit the CPT-mediated transport of lipids into the mitochondria in skeletal muscle, primarily by increasing intracellular malonyl-CoA concentrations (86). At an individual level obesity can be attributed to a sustained, positive energy balance (151). There is evidence in humans that when endogenous carbohydrate stores are increasingly utilised to provide energy for metabolism, appetite and energy intake are stimulated, with a higher rate of carbohydrate utilisation acting as the biological cue to replenish glycogen (17). It is therefore possible that a suppression of lipolysis and lipid utilisation in obesity, may increase the likelihood of a positive energy balance occurring via pathways relating to increased carbohydrate utilisation. Some indirect support for this theory comes from findings that fasting insulin concentrations predict body mass gains in Pima Indian children (152) and that adipose-tissue specific insulin receptor knockout mice show protection from obesity (153, 154). In mice, the genetic prevention of hyperinsulinemia during a high-fat diet also increases *uncoupling protein 1* expression in adipose tissue and energy expenditure, which may also explain why these mice were protected against obesity (155). Cause-and-effect relationships between hyperinsulinemia, insulin resistance and obesity are therefore complex, but common characteristics of these disease states are defects associated with lipid metabolism, a low energy flux and a low lipid flux.

In obesity an accumulation of lipid species in muscle may originate from excess supply of lipid and/or a reduced rate of lipid utilisation. The intramuscular lipid content increases after a TAG and heparin infusion (increased lipid supply) and this can impair insulin sensitivity (156). There is also evidence of blood NEFA concentrations being increased in humans with *versus* without obesity (increased lipid supply), but these differences are not always present and/or are small and appear to be unrelated to fat mass (69). This is because with an increasing fat mass, the release of NEFA per kg of adipose tissue is reduced, due to increased presence of hyperinsulinemia and a reduced blood flow to the adipose tissue (69).

Any accumulation of lipid species in skeletal muscle may also arise due to defects relating to lipid utilisation. A reduction in mitochondrial oxidative capacity of ~ 30% has been reported in skeletal muscle from humans with obesity and increased insulin-resistance *versus* healthy humans (90, 93). The mitochondrial oxidative capacity of skeletal muscle depends on both the mitochondrial content and the functional capacity of existing mitochondria. A reduction in mitochondrial density of ~ 40 % has been reported in muscle from humans with obesity *versus* lean, insulin-sensitive controls (157), but this is not always clear (100). Nonetheless, a reduction in electron transport chain (ETC) activity (157) and decreased rates of ATP synthesis in the mitochondria (158) have also been shown in muscle from humans with obesity and insulin-resistance, even if normalised to mitochondrial density, suggesting the presence of a functional defect. Muscle sampled from humans with obesity or T2D also typically has a reduced activity of CPT1, citrate synthase (CS), cytochrome c oxidase subunit I (COX1; a subunit of respiratory complex IV of the ETC) and *β*-hydroxyl-coenzyme A-dehydrogenase (*β*-HAD) compared to muscle from healthy humans (93, 159). These are enzyme markers for the capacity for lipid transport into the mitochondria, the TCA cycle, the ETC and for *β*-oxidation respectively. More evidence that mitochondrial deficiencies may have a role in the onset of insulin resistance is that decreasing peroxisome proliferator activated receptor γ co-activator 1α levels (PGC1α; a master regulator of mitochondrial biogenesis) in skeletal muscle increases insulin resistance (160). As such, mitochondrial defects may increase the likelihood of lipid accumulation in skeletal muscle and help explain a link between obesity and insulin resistance.

However, mitochondrial defects may occur secondary to a development of insulin resistance in obesity (161). Studies have shown that feeding mice a high-fat diet (162) or raising plasma NEFA concentrations (163) can increase the content of mitochondrial enzymes in skeletal muscle and the capacity of muscle to utilise lipids, despite the development of insulin resistance. Similarly, if mice are grown to have a deficiency of PGC-1α in skeletal muscle, mitochondrial enzyme activity and the capacity of muscle to utilise lipids is reduced, but glucose tolerance can be improved (164). However, in these studies the mitochondrial capacity of the

rodent muscle was often reduced to the extent that cellular ATP concentrations decreased (161). This increased intramuscular AMPK activation, which may have improved glucose tolerance by increasing GLUT4 translocation, which might not occur with a less severe mitochondrial defect (165). Nonetheless, mitochondrial deficiencies and insulin resistance might be traits of obesity without a causal link. A low energy and/or lipid flux due to physical inactivity (166) might also explain a link between obesity and an increased presence of peripheral insulin resistance. A regular utilisation of lipids derived from the adipose tissue has been linked to better metabolic health (167, 168) and decreasing lipid utilisation during exercise blunts changes in the expression of many genes relating to insulin signalling and glucose disposal in the adipose tissue (14). Similarly, stimulating IMTG utilisation has been associated with improved insulin sensitivity (169). Thus, taken together, current evidence suggests that defects relating to lipid metabolism contributes to the onset and/or development of peripheral insulin resistance in human obesity and that this impairment may be explained by defects relating to the mitochondria and/or a reduced turnover of lipid pools in skeletal muscle and the adipose tissue.

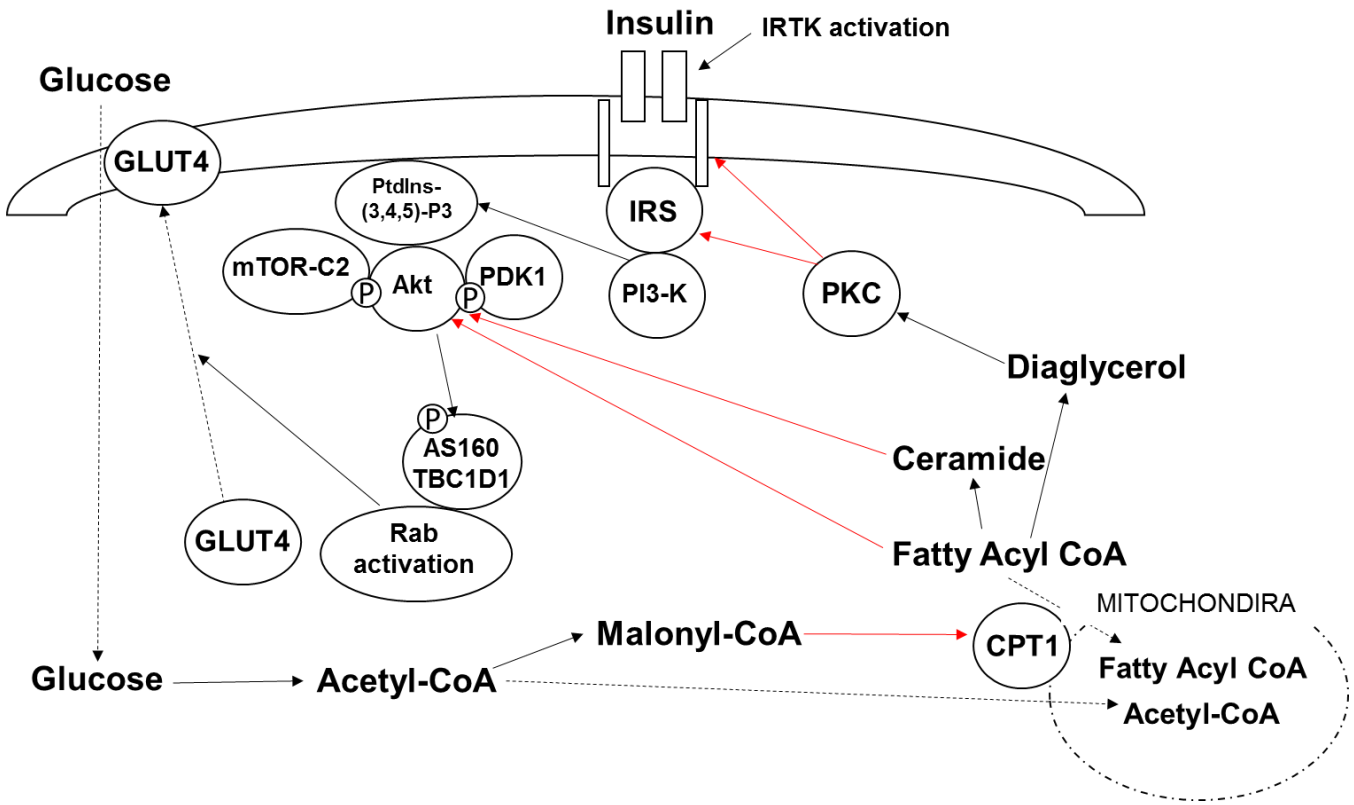


Figure 1.8. Pathways by which an accumulation of lipid metabolites may interfere with insulin signalling in skeletal muscle. Increased diacylglycerol (DAG) concentrations heightens the activation of protein kinase C (PKC). Ceramide and long chain fatty acyl-CoA have also been proposed to inhibit downstream proteins within the insulin signalling cascade. In this figure red arrows denote an inhibitory effect (on insulin signalling) Drawn using information provided in (32, 84, 86).

Abbreviations:

GLUT4 = glucose transporter 4
IRTK = insulin receptor tyrosine kinase
IRS = insulin receptor substrate
PI3-K = phosphoinositide 3-kinase
PtdIns-(3,4,5)-P3 = phosphatidylinositol (3,4,5)-trisphosphate
PDK1 = 3-phosphoinositide-dependent kinase-1
mTOR-C2 = mammalian target of rapamycin complex 2
Akt = protein kinase B
AS160 = Akt substrate of 160 kDa
CPT1 = carnitine palmitoyltransferase I
PKC = protein kinase C

LIFESTYLE MODIFICATIONS

In order to reduce rates of obesity without surgically removing adipose tissue, a negative energy balance must be created and sustained, with energy expenditure exceeding energy intake. If impairments relating to lipid metabolism and/or a low lipid flux are important in the development of insulin resistance, interventions that also increase the capacity of skeletal muscle for utilising lipids and/or stimulate a frequent turnover of endogenous lipid pools should also prove beneficial in the alleviation or prevention of metabolic diseases including T2D. Impairments in insulin signalling in skeletal muscle do not seem to be explainable by mutations in the *insulin receptor* or *GLUT4* genes in humans with obesity or T2D (170-172). Mutations in the *HK* and *glycogen synthase* genes can occur, but these are also uncommon and are not related to insulin sensitivity or glycogen synthase activity (173-176). Importantly, peripheral insulin resistance and mitochondrial defects associated with obesity can be (at least partially) reversed with lifestyle changes.

Dietary modifications

Sustained periods of dietary calorie restriction, when energy intake is reduced by 15-40 %, can facilitate body and fat-mass losses and increase peripheral insulin sensitivity (177). A large body of literature supports the benefit of this approach for improving blood glucose control, reducing inflammation in the adipose-tissue and improving blood lipid profile, across a range of populations (178). Long-term calorie restriction can also alleviate lipid accumulation in skeletal muscle, but this effect is greater if the same weight loss is achieved through exercise in humans with obesity (179). Moreover, regular exercise (and not just weight loss) may be required to alleviate some of the defects relating to intramuscular lipid metabolism in obesity. For example, when insulin-resistant humans with obesity are allocated to diet-induced weight loss or the same weight loss but via exercise and diet, only the exercisers experienced clear increases in the intramuscular mitochondrial-content or -enzyme activity (180). As such, if defects relating to lipid metabolism are important for the onset or development of insulin resistance in obesity then exercise, and not just dietary modifications, might be a more effective strategy.

Another approach that has been studied in the context of obesity and metabolic disease is adjusting the macronutrient composition of the diet, either with or without a reduction in energy intake. One of the main rationales for this approach is the proposed role of hyperinsulinemia in the aetiology of obesity and T2D and that insulin is mainly secreted in response to carbohydrate ingestion. Ingesting a low carbohydrate (< 2.5 $g \cdot kg^{-1} \cdot d^{-1}$), relatively high fat (~ 70 % of kcal) diet during exercise training increases the capacity of skeletal muscle for utilising lipids over just two weeks and has also been associated with an increased activation of lipid metabolism proteins in skeletal muscle in healthy men (181). However, if exercise is not performed, a high-fat, low carbohydrate diet can increase the IMCL content of skeletal muscle and, in some cases, impair insulin sensitivity (182). Although a carbohydrate-restricted diet can increase lipid utilisation (183, 184) this may occur at a slower rate than the rate of lipid flux into muscle, thus resulting in an increased accumulation of lipid metabolites and disrupted insulin signalling (128). Thus, when improving insulin sensitivity is a goal for an intervention, replacing dietary carbohydrate with dietary fat may not be the most efficacious strategy, especially if exercise is not performed. This research has been examined in detail elsewhere (185), and it is proposed that altering the macronutrient composition of the diet does not induce clear metabolic benefits for glycaemic control, blood lipid profiles or greater weight loss beyond those achieved with caloric restriction.

Whilst creating a negative energy balance through a reduction in energy intake can be an effective *short-term* strategy for managing the metabolic dysfunction associated with obesity, a consistent body of evidence also suggests that weight loss is often not sustainable in the *long-term*, with evidence of weight regain when assessed at 1-2 years post-intervention (186). Compensatory metabolic and/or behavioural adaptations can occur, including reductions in energy expenditure attributable to a decrease in the resting metabolic rate (adaptive thermogenesis) and changes to the secretion of appetite-regulating hormones (as discussed in **Chapter 5**). This is in line with the thrifty gene theory, which speculates that the body defends energy balance and fat stores, because our genotype was primarily selected in a hunter-gatherer period, when efficient energy storage during periods

of energy abundance and energy conservation during periods of energy shortage would favour long-term survival (186). It is proposed that following weight loss, neuroendocrine signals including low systemic leptin and insulin concentrations from the periphery convey messages regarding energy depletion to the brain, which stimulates appetite and enhances metabolic efficiency. Along with a loss of body- and fat-free mass and a decreased thermic effect of feeding, this narrows the gap between energy intake and expenditure (187). This is reflected in a meta-analysis which showed that for weight loss, a plateau often occurs at ~ 6 months, before regressing to baseline in the ensuring years with only some of the weight loss retained (188). Importantly, it has also been suggested that being regularly physical active might aid weight loss maintenance (189, 190). This is because exercise can alter the nutrient and neuroendocrine signals that feedback to the brain to regulate appetite and energy balance and because exercise can preserve fat-free mass during weight loss (191). This also supports the role of exercise as a *long-term* preventive strategy for obesity and associated metabolic diseases.

Exercise

A sedentary lifestyle is considered to be a primary risk factor for T2D and CVD (28, 192) and exercise is recommended as a strategy to prevent and treat T2D (193). Exercise guidelines suggest that exercise should be performed regularly at 40-70 % $\dot{V}O_{2\ peak}$ (peak oxygen uptake), for a minimum of 150 min·week^{-1}. This is grounded on findings that both an acute bout of endurance-type exercise and exercise training can increase blood glucose control and insulin sensitivity in humans with obesity or T2D (194, 195). This insulin sensitising effect of exercise training can also be superior to those observed in response to drugs or insulin therapy (196, 197). The beneficial effects of exercise on blood glucose control and insulin sensitivity is attributed to two main effects: an *acute* phase which is during and immediately after each bout of exercise performed and the enduring *training* adaptations that then develop in response to repeated exercise bouts. In addition, regular exercise training also increases the total amount of time that is spent in the *acute* phase, thus enhancing the metabolic benefits of training (198).

During exercise there is a marked increase in energy expenditure above rest and blood glucose provides an important fuel source for active skeletal muscle (199). Each bout of exercise activates contractile pathways in the exercising muscle, which (and independently of insulin) translocate the glucose transporter, GLUT4, to the plasma membrane and t-tubules for increased transmembrane glucose transport (200). This is largely due to the increased activation of AMPK and calcium-mediated pathways involving Ca^{2+}/calmodulin-dependent protein kinase kinases (CAMKKs) and serine/threonine kinase 11 (LKB1) (**Figure 1.9**) as well as other cellular stressors, including increased nitric oxide and reactive oxygen species (ROS) production (198, 200). Importantly, even when insulin-stimulated glucose uptake is impaired, the exercise-induced glucose uptake response can remain intact (201). In line with that finding, the pharmacological activation of AMPK stimulates glucose uptake in non-human primates and mice displaying insulin resistance (202). Whilst not a focus of this review, each bout of exercise also increases the secretion of circulating factors such as apelin and IL-6 that act in an autocrine and/or paracrine fashion and have other benefits for metabolic health, further supporting a role of exercise over other treatments for T2D (198).

After exercise there is also an increase in insulin sensitivity for ~ 48 h in skeletal muscle (198). Increased insulin sensitivity in the exercised muscle can manifest as higher *whole-body* insulin-stimulated blood glucose disposal rates, although this insulin-sensitising effect of a single bout of exercise (at the whole-body level) is not always apparent in metabolically healthy, physically active humans (198). Therefore, the acute insulin-sensitising effect of exercise is best thought of as a localised effect, mostly restricted to the muscle(s) that were active during exercise (198). Following exercise, meal-derived glucose is largely used to restore muscle glycogen (203). The rate-limiting step in this process is the conversion of uridine diphosphate-glucose to glycogen (**Figure 1.3**). This reaction is catalysed by glycogen synthase, which is increasingly activated after exercise (204). However, net glycogen formation can remain elevated after exercise, even when glycogen synthase activity has returned to basal levels (205). This suggests that increased glycogen synthase activity cannot completely explain an increased capacity of

exercised muscle for disposing of blood glucose. The plasma membrane GLUT4 content also returns to basal levels in ~ 2 h of exercise cessation (206) despite a transient increase in muscle insulin sensitivity for ~ 48 h after exercise in healthy humans (207) and for ~ 15 h in humans with T2D (208). After the immediate post-exercise period, an increased capacity of skeletal muscle for glucose disposal can be largely explained by the GLUT4 response to subsequent insulin secretion (209). Indeed insulin-stimulated GLUT4 translocation is increased *in vitro* if the muscle has been pre-treated with insulin (without differences in Akt activation) compared to muscle that was not pre-treated (210). Prior exercise can augment this pre-treatment effect, as insulin and exercise stimulate the translocation of GLUT4 via different signalling pathways and possibly from distinct intracellular stores (211). Although insulin and exercise active different proximal signalling pathways in skeletal muscle, they do converge at distal proteins (i.e. AS160 and TBC1D1), with exercise acting via an AMPK signalling cascade (**Figure 1.9**).

People who regularly exercise normally have lower blood insulin concentrations during fasting, glucose infusions (212) and after oral glucose ingestion (213) than sedentary controls. This is supported by evidence that exercise training increases insulin sensitivity, even after a week of detraining, which suggests an adaptation and not just an acute effect of exercise (214). In humans with impaired glucose tolerance, regular exercise can lower blood glucose concentrations, although this depends on the degree of glucose intolerance at baseline and the specific training protocol used (215). Mechanisms behind the increases in insulin sensitivity with exercise training are multifactorial (200). Some *in vitro* studies report a higher expression of IRS proteins following exercise training, but this is not always clear (200). Instead, increases in insulin sensitivity with exercise training are more likely to be attributable to altered downstream insulin signalling (216, 217), which may be due AMPK signalling (218). Increased AMPK activity after each exercise bout performed may account for the higher *GLUT4* expression and protein content in exercised muscle after training (165), which has been linked to an increased capacity for blood glucose disposal (219, 220). Exercise training also increases intramuscular capillary density, increasing the capacity for the delivery of glucose

and insulin to skeletal muscle (221). Although intramuscular blood flow does not normally limit blood glucose disposal in healthy humans, this effect of exercise may be important in humans with obesity, where a reduction in skeletal muscle permeability has been shown (222). Alterations in body composition such as a reduced fat mass and an increased fat-free mass can also occur (223) but are not required for changes in insulin sensitivity with exercise training (224).

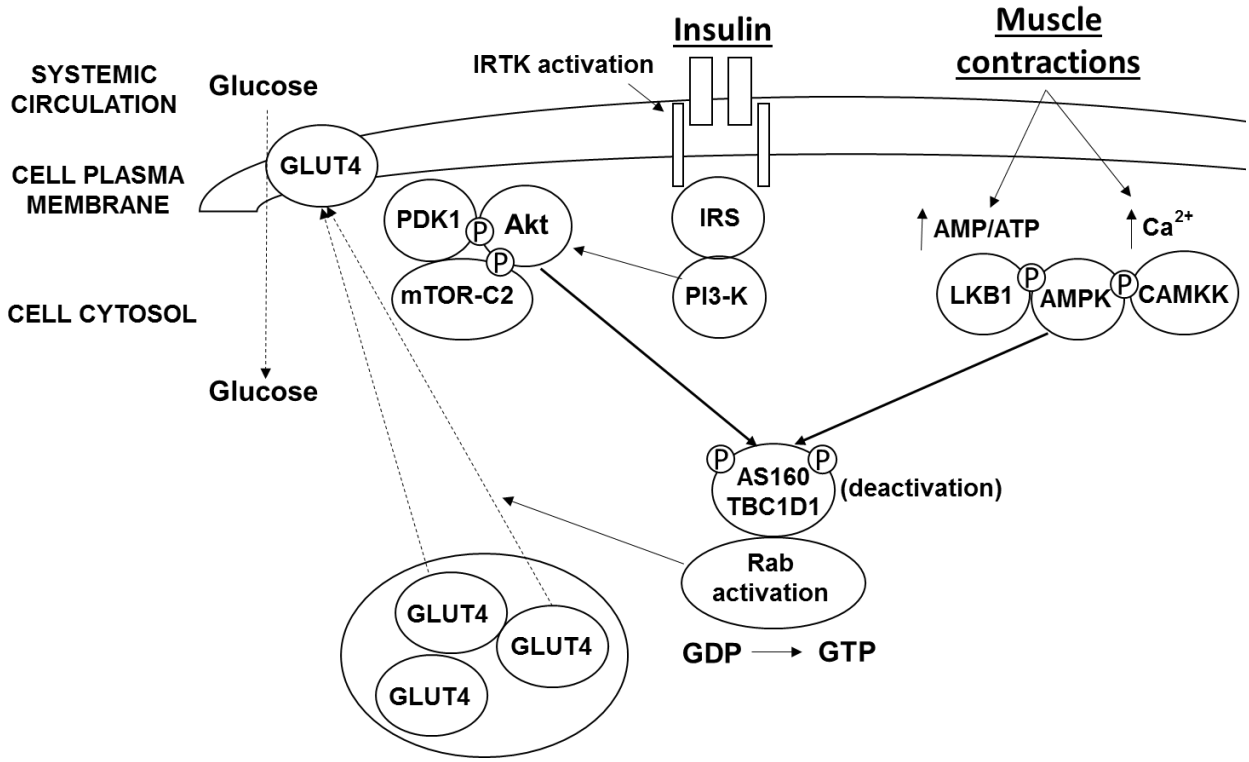


Figure 1.9. Pathways by which insulin and muscle contractions (exercise) result in increased GLUT4 translocation. Redrawn from a figure produced in (51).

Abbreviations:
GLUT4 = glucose transporter 4
IRTK = insulin receptor tyrosine kinase
IRS = insulin receptor substrate
PI3-K = phosphoinositide 3-kinase
PDK1 = 3-phosphoinositide-dependent kinase-1
mTOR-C2 = mammalian target of rapamycin complex 2
Akt = protein kinase B
AS160 = Akt substrate of 160 kDa
AMPK = 5' AMP-activated protein kinase
CAMKK = alcium/calmodulin-dependent protein kinase kinase 2
LKB1 = liver kinase B1 or serine/threonine kinase 11 (STK11)

Adaptive responses to exercise training may also be specifically related to lipid metabolism. With each exercise bout, there is an acute increase in the turnover of intramuscular triglyceride (IMTG) and adipose-tissue derived NEFA (225, 226). A regular utilisation of NEFA derived from the adipose tissue has been associated with an increased ability to buffer subsequent lipid flux (167, 168). The regular utilisation of IMTG has also been positively associated with insulin sensitivity, potentially due to an alleviation of the toxic lipid intermediates (169). Importantly, humans with obesity can stimulate lipid metabolism during exercise and whole-body lipid utilisation rates can even be higher in men (227) and women (228) with *versus* without obesity, when matched for cardiorespiratory fitness (**Figure 1.10**). Thus, if the regular turnover of endogenous lipid pools is important for metabolic health, then any effect of exercise training on insulin sensitivity may be partially accounted for by spending more time in the '*acute phase*' of exercise (198).

In addition, following exercise training there is also evidence of an increase in the capacity of skeletal muscle to utilise lipid substrates subsequently (10, 229, 230). PGC1-α is a protein that may be important in this regard, because it is proposed to be the master regulator of mitochondrial adaptations (231, 232) and can be activated by AMPK (233). With a repeated increase in the activity of AMPK and other cell kinases with regular exercise training, mitochondrial volume, enzyme activity (234-236) and the content of proteins involved in lipid metabolism is increased (237, 238). This may reduce the likelihood of toxic lipid intermediates accumulating in skeletal muscle and contribute to increases in insulin sensitivity (200). In support of this theory, in one study a strong predictor (~ 50 % of the variance), for changes in insulin sensitivity following 16 weeks of exercise training in humans with obesity was increases in lipid utilisation during rest (239). It has also been shown that humans who regularly exercise store intramuscular lipid in intramyofibrillar droplets, but humans with T2D have large subsarcolemmal lipid droplets (240). Exercise training may therefore increase the storage of IMTG in pools that are more easily accessible for metabolism without causing lipotoxicity. Similar results have been shown with regards to muscle glycogen (241). Together

with increments in mitochondrial oxidative capacity, this effect of exercise training may help alleviate any lipid-related defects associated with metabolic diseases.

The total volume of exercise performed (duration x intensity x frequency) is a key determinant of the magnitude of many adaptations to exercise training (242). However, many people fail to achieve current exercise guidelines, partly due to a perceived lack of time (6, 243). For these people, and for people who cannot increase the intensity or duration of exercise beyond a certain limit (for example due to a low level of fitness) any optimisation of exercise efficacy is important. In addition, some individuals may not always achieve the full potential benefits of exercise training. For example, the HERITAGE family study reported that on average exercise training increased insulin sensitivity, but 40 % of participants showed negligible changes to- or decreased-insulin sensitivity (7). This inter-individual variability for postprandial insulinemia following exercise training has also been shown to be greater than that of a control group (244). This suggests that some of the variability is true inter-individual variability and not intra-individual variability and/or measurement errors (245). These studies did not control for the nutrient status of participants before exercise. If a regular turnover of endogenous lipid pools or adaptations relating to lipid metabolism are important for changes in metabolic health, this lack of a control for nutrient-exercise interactions may partly explain any inter-individual variability in response to the exercise training.

SUBSTRATE METABOLISM DURING EXERCISE

The increase in energy demands in the transition to exercise from rest is met by increased carbohydrate and lipid oxidation, but the relative contribution of these substrates depends on the exercise intensity (225). Henceforth, low- moderate- and high-intensity exercise describe physical activity that is performed at < 50 %, 50-65 % and > 65 % $\dot{V}O_2$ peak respectively. The research used to inform this part of the literature review was also mostly conducted in healthy, fasting humans. Four main energy sources are utilised by skeletal muscle for energy metabolism during exercise, as protein utilisation does not make a meaningful contribution in most circumstances (246). For carbohydrates, these are muscle glycogen and

blood glucose (derived from the liver) and for lipids these are intramuscular triglyceride (IMTG) and blood NEFA derived from the adipose tissue (225, 226). During fasting and at low exercise intensities a large proportion of the energy for metabolism can be derived from lipids (**Figure 1.11**). In the transition from rest to exercise the increased net mobilisation of NEFA from the adipose tissue is partly due to the ~ 3-fold increase in blood catecholamine concentrations (247). Visceral adipose tissue is very responsive to adrenergic activation, but subcutaneous adipose tissue is a greater contributor to plasma NEFA flux during exercise, due to its larger size (247). Exercise also increases plasma IL-6 concentrations (248), which may contribute to the exercise-induced increase in lipolysis in the adipose-tissue (77). When the exercise intensity increases from low to moderate intensity, lipid utilisation increases and, as an absolute rate, is highest at 45-65 % $\dot{V}O_2$ peak (249). This is due to a greater rate of net IMTG utilisation, as the mobilisation of NEFA from the adipose tissue decreases with this increment in exercise intensity (250). As such, for fasted-state moderate-intensity exercise, NEFA and IMTG are roughly equal contributors to whole-body lipid utilisation (**Figure 1.11**). In the transition from moderate- to high-intensity exercise lipid utilisation then markedly decreases (249) and to account for this, whole-body carbohydrate utilisation rates are increased (225, 226). During low intensity exercise, a greater proportion of the carbohydrate utilised for metabolism is from blood glucose, which is largely derived from the liver when fasting (67). However, during high intensity exercise muscle glycogen is increasingly utilised to provide energy for metabolism and can represent ~ 75-85 % of whole-body carbohydrate utilisation (67).

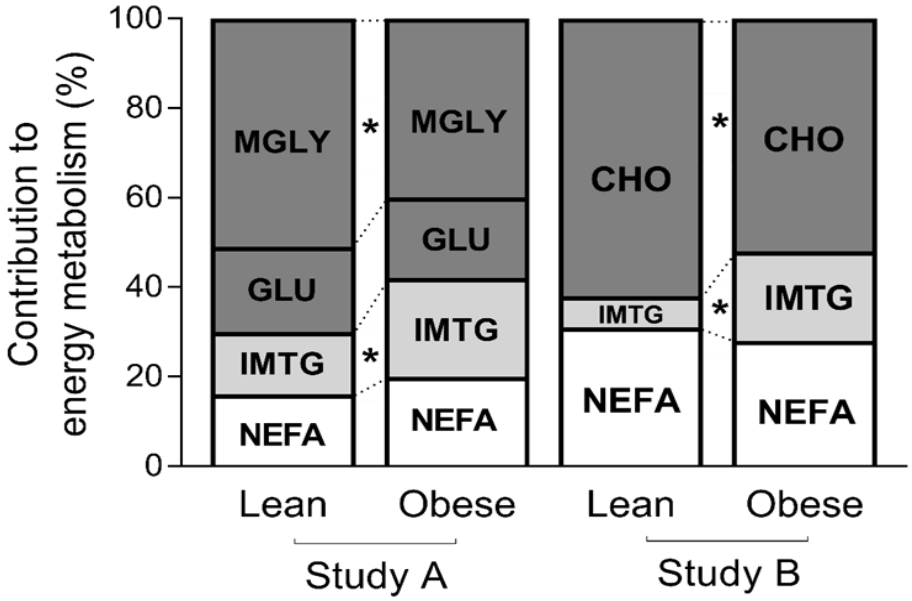


Figure 1.10. The contribution of substrates to energy metabolism during exercise in humans with/without obesity. Lipid utilisation represents plasma non-esterified fatty acids (NEFA) and intramuscular triglyceride (IMTG & plasma lipoproteins). Carbohydrate utilisation represents plasma glucose (GLU) and muscle glycogen (MGLY). **Study A** was 60 min of fasted cycling at 50 % $V02_{peak}$ in men matched for fitness levels (227). **Study B** was 90 min of fasted cycling at ~ 55 % $\dot{V}O2_{peak}$ in women matched for cardiorespiratory levels (228) * is $p<0.05$.

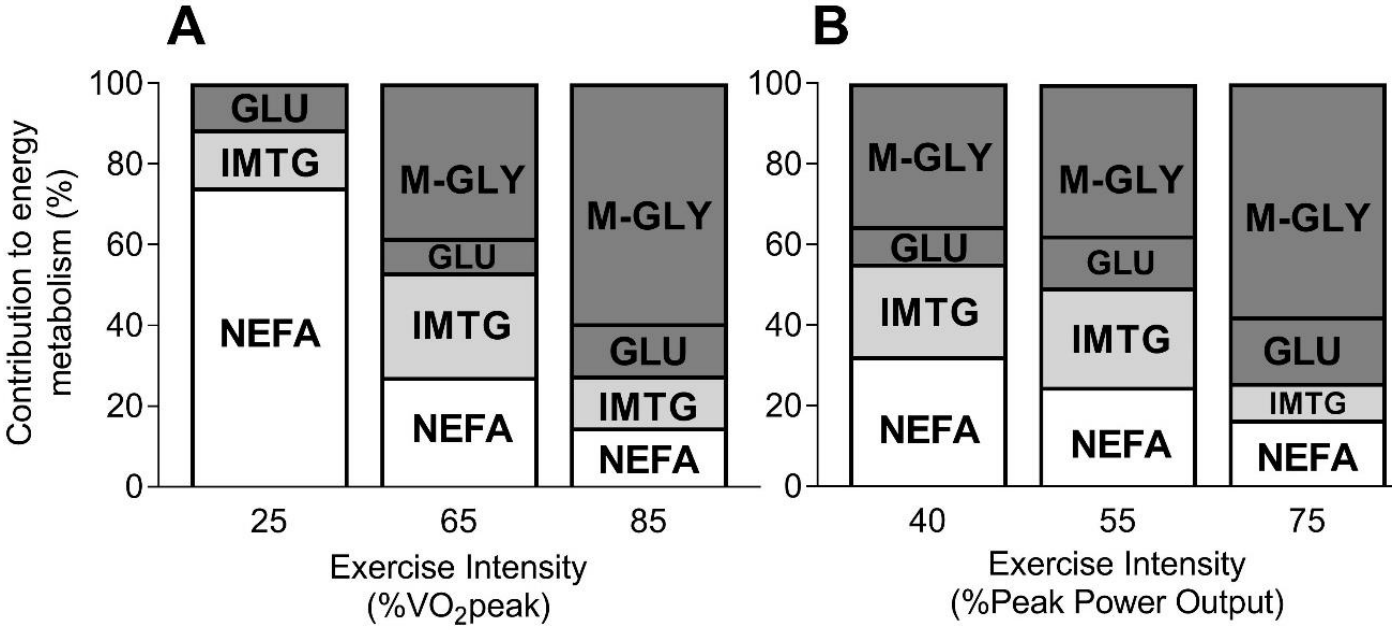


Figure 1.11. A contribution of substrates to energy metabolism during exercise in (healthy) humans without obesity**.** Lipid utilisation is plasma non-esterified fatty acids (NEFA) and intramuscular triglyceride (IMTG & plasma lipoproteins) and carbohydrate utilisation represents plasma glucose (GLU) and muscle glycogen (MGLY). Drawn using data reported in (225) for **Study A** and (226) for **Study B**.

Limitations to lipid utilisation

Relative to carbohydrates, lipid stores in the body are vast [> 100,000 kcal for a 75 kg man without obesity compared to < 3000 kcal for carbohydrates (251)]. Indeed, carbohydrates stores typically represent ~ 1-3 % of the energy in fat stores, even in exercise-trained humans who have an increased capacity to store glycogen. However, during exercise lipids are not always the greatest contributor to energy metabolism. The utilisation of blood lipids for metabolism may be at least partly regulated by lipid availability, as when blood NEFA concentrations are reduced with nicotinic acid analogues (252) or elevated using TAG and heparin infusions (253) lipid utilisation in skeletal muscle is decreased or increased, respectively. Exercise can stimulate lipolysis in the adipose tissue by increasing adrenergic *β*-receptor activation (254) and for low-intensity exercise, splanchnic blood flow increases by > 4 fold *versus* rest (255), facilitating a higher net NEFA mobilisation. However, at higher exercise intensities, splanchnic blood flow is reduced as respiratory and exercising muscles are increasingly prioritised (255). As a result, NEFA become trapped in adipose tissue and systemic concentrations increase when blood flow is redistributed post-exercise (and at a greater rate than glycerol), which suggests that lipolysis is not limiting NEFA mobilisation (250).

In theory, another potential limiting factor for lipid utilisation during exercise is the transport of NEFA into the myocyte, which is facilitated by fatty acid transport proteins, although passive diffusion of NEFA across the plasma membrane is possible (**Figure 1.7**). However, lipids can accumulate in skeletal muscle during high-intensity exercise, suggesting that transport across the plasma membrane is not limiting their utilisation (256). In addition, IMTG do not need to cross the endothelium or the sarcolemma, so transmitochondiral membrane is a more likely site for any transport-related limitation to lipid utilisation. In this process, long chain fatty acid acetyl-CoA (from an esterification of fatty acids and CoA with ATP in the cytosol), forms a complex with L-carnitine which is catalysed by CPT-1 (**Figure 1.7**). The complex is transported into the mitochondria, where CPT-2 can then catalyse its transesterification back to free-carnitine and long chain fatty acid acetyl-CoA. As intramuscular free-carnitine concentrations are decreased during

high-intensity exercise, this may explain the decrease in rates of lipid utilisation (226). Finally, lipids have a lower energy production rate per unit of time and require more O_2 to yield a given amount of energy *versus* carbohydrates (30). As such, the oxidative capacity of the mitochondria in skeletal muscle is a potential limiting factor for lipid utilisation (88). When oxidative metabolism cannot meet the energy demands of exercise, anaerobic glycolysis is increased (257). Along with a decreased intracellular pH, this can inhibit the CPT-transport system (258).

Variation in lipid utilisation during exercise

Rates of lipid utilisation during fasted-state exercise also shows some apparent inter-individual variation. If untrained men exercise at a moderate intensity during fasting, the RER can range from 0.83 to 0.95 (259). Similarly, the measured RER of trained cyclists has been reported to range from 0.72 to 0.93 during fasting at rest, with a similar variation persisting during exercise (260). Training status, fat- or fat-free mass, physical activity and sex differences appear to explain a small amount (~ 35 %) of this variance (e.g. from 0.18 to 1.01 $g \cdot min^{-1}$ (97)]. Fasting before laboratory-based exercise trials is a common procedure to help control for baseline metabolic status, but this may also preclude the application of results to situations most representative of daily living. For many humans in developed countries, exercise is often performed in the evening (261). In concert with a high carbohydrate diet consumed by many people living in developed countries (186), it follows that most exercise sessions are undertaken after eating carbohydrates. Since at least 1920, we have known that meal timing in relation to exercise alters metabolism during exercise (262). Indeed, consuming carbohydrates 30-180 min before exercise lowers whole-body lipid utilisation and increases carbohydrate utilisation relative to when exercise is performed in a fasted-state (**Figure 1.12**). The highest absolute rate of lipid utilisation during exercise in a fasted-state and the biggest difference in lipid utilisation with exercise performed before *versus* after nutrient ingestion occurs during moderate-intensity exercise (**Figure 1.13**).

A decrease in lipid utilisation during exercise that is performed after consuming carbohydrates is irrespective of sex, body composition, training status or the time

between the meal [if < 6 h (263)] and the exercise (264) and is partly due to insulin supressing NEFA mobilisation from the adipose tissue, by inhibiting lipolysis and stimulating NEFA re-esterification (10). A higher intramuscular glucose flux and decreased HSL activity (due to a lower blood catecholamine: insulin ratio), might also inhibit IMTG utilisation with exercise performed in a carbohydrate-fed state (265, 266). An overnight fast typically lowers liver glycogen concentrations by around 40 % [350 ± 18 mmol·L^{-1} 4 h after a 60 % carbohydrate and 650 kcal meal compared to 207 ± 22 mmol·L^{-1} the morning after (36)], as liver glycogen helps to maintain blood glucose concentrations during the overnight fast (30). Two hours after a carbohydrate-rich breakfast is consumed, the liver glycogen content can then be increased by > 20 % (36). As such, a lower hepatic glycogen content with exercise performed during morning fasting (*versus* after breakfast) might also increase adipose tissue lipolysis via liver-brain-adipose tissue signalling (267).

Consuming carbohydrates before and/or during exercise can delay the onset of fatigue by sparing muscle glycogen stores (268) and can improve performance (269). Theoretically, this may enhance some adaptations to exercise by allowing training to be performed at a higher intensity or maintained for longer, increasing the overall exercise dose (242). However, a lack of time is often cited as a main barrier to exercise (6, 243), so for many people, fatigue due to muscle glycogen depletion is not likely to be limiting to the exercise dose performed, as glycogen depletion only occurs after ~ 2 h of moderate-intensity exercise (225). For these people augmenting exercise adaptations when the exercise is time- and intensity-matched will be of greater importance. Instead, if increasing lipid flux is important for alleviating insulin resistance in obesity, eating carbohydrates before and/or during exercise may blunt the health benefits gained from fasted-state exercise.

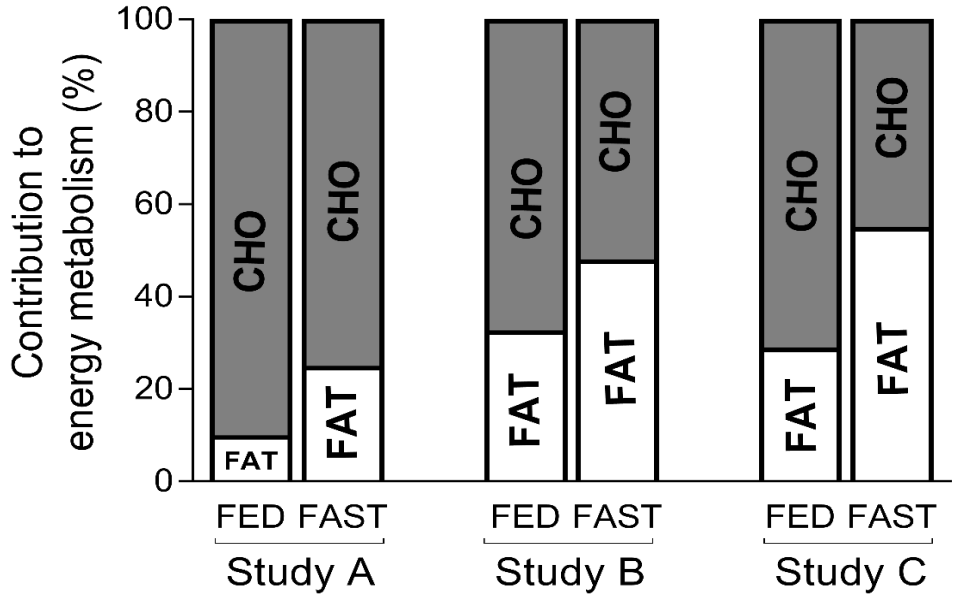


Figure 1.12. Substrate utilisation during exercise performed before (FED) *versus* after (FAST) consuming breakfast. In **Study A** participants cycled for 30 min at 60 % $\dot{V}O2_{peak}$ after a 12-h fast or 3 h after a carbohydrate rich breakfast (249), in **Study B** participants ran for 60 min at 60 % $\dot{V}O2_{peak}$ after a 12-h fast or 2 h after a carbohydrate rich breakfast (15) and in **Study C** participants cycled for 60 min at 55 % $\dot{V}O2_{peak}$ after a 12-h fast or 1 h after a carbohydrate rich breakfast (10).

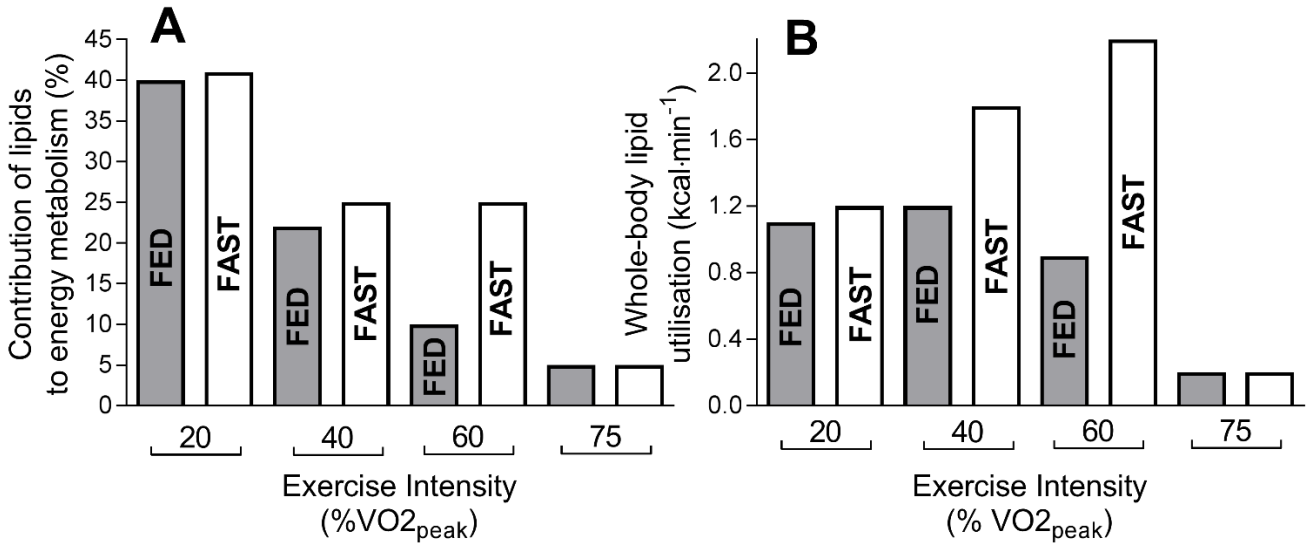


Figure 1.13. The contribution of lipids to energy metabolism (**A**) and whole-body lipid utilisation (**B**) during cycling exercise performed after breakfast (FED) or fasting (FAST). Drawn using data provided in (249).

BREAKFAST, EXERCISE AND GLYCAEMIA

Glycaemia during- and post-exercise (acute)

The focus of this review is nutrient-exercise interactions and glucose metabolism *after* exercise. However, consuming *versus* omitting breakfast before exercise also alters glycaemia *during* exercise (270). Comparing this research is difficult due to differences in study designs, including the timing and the composition of meals, but most studies have compared moderate intensity endurance-type exercise before *versus* after (60-120 min) consuming a meal containing ~ 100 g of carbohydrate. If exercise is performed pre-meal, blood glucose concentrations during the activity are similar to- or are even slightly higher than rest, although lower blood glucose concentrations have been shown *versus* rest after > 60 min of fasted-state exercise (270). Despite similar blood glucose concentrations during exercise *versus* rest, blood glucose flux is still increased during exercise in the fasted state (271). Consuming *versus* omitting breakfast prior to exercise further increases rates of whole-body blood glucose appearance and disposal during exercise, with increased glucose oxidation contributing to higher whole-body carbohydrate utilisation rates (272). Exercising immediately after a high-glycaemic index, carbohydrate-rich meal can induce acute hypoglycaemia as exercise and insulin (secreted in response to the meal) both stimulate GLUT4 translocation and blood glucose disposal (273). However, if moderate-intensity, endurance-type exercise is undertaken 30- to 120-min after a carbohydrate-rich meal, blood glucose excursions from the meal are lowered relative to when exercise is not performed beforehand (274, 275), which might reduce CVD risk by alleviating hyperglycaemia, if this stimulus is repeated regularly (276-278).

Each bout of endurance-type exercise typically increases insulin sensitivity in the exercised muscle (194). However, blood glucose concentrations following *post-exercise* meals are not always lowered relative to rest (279). This is because an exercise-induced increase in postprandial blood glucose disposal rates can be offset, or even superseded, by higher endogenous or meal-derived blood glucose appearance rates (279). During rest, consuming a carbohydrate-rich meal lowers blood glucose concentrations in response to subsequent meals (280, 281). The

underlying mechanisms for this second-meal effect include delayed gastric emptying [with a 50 % increase in the time to clear 50 % of the stomach content at the second meal (282)], which may be due to higher GLP-1 secretion (283). GLP-1 also stimulates insulin secretion (40) and along with any priming of the pancreatic *β*-cells due to prior insulin secretion, this can potentiate early phase insulinemia after the second meal (284). Any potentiation of the insulin response to feeding could further suppress HGP and increase peripheral blood glucose disposal rates at the second meal (285). An increase in blood glucose disposal rates may also be attributable to a priming of skeletal muscle by prior insulin exposure (210). Finally, insulin-stimulated hepatic glucose disposal can also be increased by prior exposure to insulin in dogs (286), so if this occurs in humans, a priming of the liver might also contribute to increased blood glucose disposal rates and/or a greater supression of HGP at subsequent meals consumed at rest.

When breakfast is consumed before 60 min of running at 60 % $\dot{V}O_2$ peak, blood glucose concentrations are higher in response to a post-exercise mixed-macronutrient meal *versus* breakfast consumption at rest (15). The postprandial area under the curve (AUC) for blood glucose was 15 % higher with breakfast consumption before exercise *versus* breakfast consumption at rest, but this difference with exercise *versus* rest was not clear when breakfast was omitted. Although confirmatory research is required, it is likely that higher postprandial blood glucose appearance rates from the meals and/or HGP after exercise with breakfast consumption before exercise was responsible for that finding. Gastric emptying rates are not clearly altered during moderate-intensity endurance-type exercise *versus* rest (287). However, performing exercise increases splanchnic blood flow at subsequent meals by ~ 15-35 % (203) and eating prior to exercise increases splanchnic blood flow during the exercise (10). This may raise blood glucose appearance rates at meals that are consumed post-exercise, especially when breakfast is consumed before the activity. Intestinal absorptive cells can become damaged during exercise due to increased splanchnic hypoperfusion (288). Splanchnic hypoperfusion is not always evident during low- or moderate-intensity exercise (10), however, higher core body temperatures, increased

mechanical stress in the GI tract and higher systemic adrenaline concentrations can also increase the intestinal damage response to exercise (289-291). This intestinal damage response to exercise may depend on the presence of nutrients in the GI tract via increased mechanical stress, which may also explain increased post-exercise blood glucose appearance rates with breakfast consumption *versus* omission before exercise (292). Finally, the liver glycogen content can be increased after breakfast consumption (36), so residual post-exercise HGP may contribute to higher postprandial post-exercise blood glucose appearance rates compared to rest, or exercise with breakfast omission. A higher muscle glycogen content decreases net muscle glucose disposal rates, due to reduced GLUT4 translocation and decreased HK activity (194). However, even if muscle glycogen concentrations were increased before exercise with prior breakfast consumption (*versus* omission) the utilisation of glycogen during exercise could, in theory, be increased in that condition (293), to the extent that post-exercise concentrations might not clearly differ. However, these mechanisms are speculative and further research is required to investigate post-exercise postprandial glucose flux with prior breakfast consumption *versus* omission. This research demonstrates that performing exercise alters the glycaemic responses to the first meal post-exercise compared to rest, and that this response may be altered by the prior provision (or omission) of nutrients. It should however also be acknowledged that studying the effects of exercise-nutrient interactions over a longer time is important as exercise increases insulin sensitivity over a period spanning many meals (294, 295).

Exercise training and glycaemia

Training studies with exercise performed before *versus* after breakfast either report no change in fasting blood glucose or insulin concentrations (265), or lower fasting concentrations after training, but with the magnitude of this change *versus* no-exercise not clearly differing between exercise groups (11, 12). However, if healthy men consume a hypercaloric (+ 30 % kcal of habitual diet) fat-rich (50 % of kcal) diet, postprandial oral glucose tolerance is improved and the $ISI_{MATSUDA}$ index of insulin sensitivity [an estimate of peripheral insulin sensitivity typically derived from an oral glucose tolerance test (296)] is increased, if regular exercise

is performed during morning fasting, but not when breakfast is consumed before- and carbohydrates consumed during-exercise (11). In that study, participants ran and cycled for 300 min·week^{-1} over 6 weeks and either had a 675 kcal (70 % carbohydrate) breakfast 90 min before- and consumed 1 g·kg^{-1} of carbohydrate during-exercise or exercised in the fasted-state (eating breakfast and the extra carbohydrate post-exercise). Relative to a non-exercise control group, the blood glucose AUC in response to an oral glucose tolerance test (OGTT) decreased if exercise was performed fasted (238 ± 22 to 173 ± 11 mmol·min^{-1}·L^{-1}; p <0.05) but not after breakfast (263 ± 43 to 250 ± 36 mmol·min^{-1}·L^{-1} p >0.05) and the $ISI_{MATSUDA}$ index also increased only if exercise was performed before breakfast. Although a high-fat diet is not most representative of the diet consumed by many people living in developed countries (186), this suggests that the timing of nutrient ingestion in relation to exercise may be important for exercise-induced changes in oral-glucose insulin sensitivity. However, it is possible that increases in body mass in the control- and exercise after breakfast-groups (and not in the exercise before breakfast group) might explain the differences in insulin sensitivity in that study. Despite this, other studies report that exercise training before *versus* after breakfast increases basal muscle glycogen concentrations (11, 12, 297). Defects associated with muscle glycogen synthesis are shown in insulin-resistant states (298) and an increased ability to store carbohydrate as muscle glycogen after exercise training has been associated with increased insulin sensitivity (294). As such, higher basal muscle glycogen contents reported following exercise before *versus* after breakfast also suggests increased insulin sensitivity in that condition.

Regularly exercising before *versus* after breakfast may offer a greater stimulus for increasing peripheral insulin sensitivity. If this is the case, the acute increase in whole-body lipid and/or IMTG utilisation (265) performed before *versus* after breakfast may be responsible. A regular utilisation of NEFA derived from the adipose tissue has been associated with an increased ability to buffer subsequent lipid flux (167, 168). Consuming breakfast prior to exercise also blunts effects seen after exercise during morning fasting for the expression of genes relating to lipid metabolism and insulin sensitivity in the adipose tissue (14). The regular

utilisation of IMTG has also been associated with increased insulin sensitivity, potentially due to an alleviation of toxic lipid species (169). However, in the work by Van Proeyen *et al.* (11), insulin sensitivity increased with exercise before breakfast, but ceramide and DAG concentrations in exercised muscle remained largely unaltered. Nonetheless, their participants were healthy, so excess lipid influx to skeletal muscle due to the high-fat diet may have been partitioned into IMTG preventing any accumulation of these lipid species (299). Indeed, the IMTG content of type I and IIa fibres in the *vastus lateralis* was increased by ~ 50 % and ~ 75 %, respectively. As the ability to synthesise IMTG is suggested to be impaired in humans with obesity (300), this IMTG-synthesis pathway may be less effective in buffering lipid flux into muscle in those populations. Regular exercise in humans with obesity has been reported to decrease the intramuscular DAG and ceramide contents and increase insulin sensitivity (301). Whether nutrient-exercise interactions alters the magnitude of this effect warrants investigation.

Manipulating aspects of carbohydrate metabolism during exercise also alters some adaptations relating to the capacity of skeletal muscle for utilising lipid substrates (**Figure 1.14**). In one study, healthy men completed knee-extensor exercise for 60 min at 75 % peak power output (PPO) over 10 weeks, with one leg training daily and the other leg twice daily every second day, with a 2-h fasted rest between the sessions (302). Ingesting 7-10 $g \cdot kg^{-1}$ of carbohydrates post-exercise favours the restoration of muscle glycogen (303) and the diet given in that study (~ 675 g) would have been sufficient to (at least partially) restore muscle glycogen in the leg training daily after each bout of exercise. In contrast, the leg training twice daily would have completed the second session with a lower muscle glycogen content. Post-intervention, the increase in the activity of CS in exercised muscle was greater in the leg training twice daily. In a design more readily applicable to a mode of exercise often performed, cyclists ate a diet providing 8 $g \cdot kg^{-1} \cdot day^{-1}$ carbohydrate and alternated daily for 6 $day \cdot week^{-1}$ between moderate- (100 min at 63 % PPO) and high-intensity cycling (8 x 5 min) or completed both sessions on the same day every second day, separated by a 2-h fast (304). After 3 weeks, rates of lipid utilisation during fasted submaximal

exercise, the maximal activity of both CS and *β*-HAD and the content of the ETC protein COX-IV in the *vastus lateralis* were increased to a greater extent in the men training twice daily (with some exercise sessions started with a low muscle glycogen content). It has since been confirmed that training with a reduced muscle glycogen content (~ 35-45 % of resting values) can increase whole-body lipid utilisation during subsequent exercise in the fasted-state and also augment mitochondrial adaptations compared to the same exercise commenced with a higher muscle glycogen content (305, 306) [although the extent to which muscle glycogen is depleted pre-exercise may be important in this regard (307)].

Although training twice daily might not be sustainable people exercising primarily for health reasons, exercise training (at a lower dose) performed before *versus* after ingesting carbohydrates also results in greater increases in the content or the *mRNA* expression of proteins in exercised muscle such as CD36 and CPT-1 that are involved in lipid metabolism, over a period of just 4-8 weeks (12, 13, 308). Markers of mitochondrial biogenesis can also be increased after exercise training before *versus* after breakfast (297) although this effect is not always evident (11). These adaptations may increase the capacity of skeletal muscle for utilising lipids which might then have implications for insulin sensitivity (309). Although exercise training before *versus* after breakfast does not always increase whole-body lipid and/or IMTG utilisation during subsequent fasted-state exercise (11, 297, 310), it should be noted that the workload of the exercise tests used to measure this were typically not adapted to account for any changes in the capacity for lipid utilisation.

The exercise-induced activation of intramuscular energy sensing pathways can be augmented when exercise is performed with a reduced relative to high muscle glycogen content (311, 312). Together with evidence of a glycogen-binding site on AMPK (313), this suggests that glycogen availability during exercise may regulate some adaptations to exercise (314). Aside from AMPK, other proteins regulate mitochondrial adaptations, such as p53, which is activated by exercise and to a greater extent when muscle glycogen availability is decreased (315). Regular exercising before *versus* after breakfast (without prior exercise to deplete

muscle glycogen) also augments mitochondrial adaptations (297) and consuming carbohydrate during exercise commenced with a reduced muscle glycogen content blunts greater increases in mitochondrial biogenesis otherwise observed (306). This suggests that adaptations to exercise with a reduced carbohydrate availability cannot be completely explained by muscle glycogen availability.

Breakfast omission before exercise results in greater net NEFA mobilisation from adipose tissue and increased lipid utilisation, relative to when carbohydrates are consumed before and/or during exercise (264). Raising (163) or lowering blood NEFA concentrations (316) increases and supresses the expression of proteins involved in intramuscular mitochondrial biogenesis, respectively. This might be because fatty acids are ligands for peroxisome proliferated activator receptors (PPARs) which alter the expression of lipid metabolism proteins in muscle (317). PPAR-δ regulates the expression of *CPT1* (318) and enzymes in the fatty acid oxidation pathway (319) and the exercise-induced increase in PPAR-δ activity can be augmented by performing the activity with a low carbohydrate availability (320). Although mitochondrial biogenesis is regulated by many transcription factors, this is largely co-ordinated by PGC-1*α*, and PPAR-δ and AMPK help to regulate PGC-1*α* expression (321). A higher intramuscular AMPK activity also stimulates GLUT4 biogenesis, which may have implications for exercise-induced changes in insulin sensitivity (165). Carbohydrate consumption during exercise inhibits the increase in *GLUT4* mRNA abundance (322) and after training, the GLUT4 content of muscle is often (11, 308) [although not always (13)] increased with exercise before *versus* after breakfast. Thus, an acute increase in the activity of energy sensing proteins and/or a higher lipid flux during each bout of exercise performed before *versus* after breakfast may stimulate key adaptations in muscle.

Finally, alterations to the phospholipid (PL) composition of skeletal muscle have been associated with obesity and insulin resistance (134). In healthy men, six weeks of daily (> 30 mins) moderate intensity exercise at 55% $\dot{V}O_2$ peak alters the PL content of skeletal muscle (323). In that study, peripheral insulin sensitivity increased by 22 % in an exercise- compared to a control-group. In the exercise

group the proportion of the saturated fatty acid - palmitic acid - in the PL pool in the *vastus lateralis* decreased and the proportion of oleic acid - an unsaturated fatty acid - was increased. That result may be important in light of associations between insulin sensitivity and the palmitic acid content of the PL pool in skeletal muscle (324). The training also decreased the total sum of *n*-6 poly-unsaturated fatty acids (PUFAs) but there was no clear change in the sum of the *n*-3 PUFAs, which resulted in a trend for a lower *n*-6-to-*n*-3 ratio in the intramuscular PL pool ($p<0.13$). However, changes to specific PUFA species must be interpreted more cautiously as they may be accounted for by errors associated with assessing the content of fatty acids that represent a small proportion of the total PL pool (134). Understanding if the changes in the PL pool were important for insulin sensitivity in that study was also difficult because insulin sensitivity was assessed 24 h after the last exercise session (so the changes may be due to an acute exercise effect).

In another study, 8 men completed single-leg training for 30 min·day^{-1} for 4 weeks at 70 % $\dot{V}O_2$ peak (325). That study did not detect clear changes in individual PL fatty acids in the *vastus lateralis* post-exercise *versus* no-exercise but the PUFA content of the PL pool tended to be higher after training ($p<0.06$) and the sum of C20-22 PUFAs was correlated with insulin sensitivity from a hyperinsulinemic-euglycemic clamp ($r=0.574$; $p=0.04$). However, these relationships were not clear mid-intervention, despite insulin sensitivity increasing from baseline in the exercise group. Since this PL remodeling was also independent of dietary intake, the reduction in the saturated fatty content of the PL pool was potentially due to an upregulation of saturated fatty acid oxidation attributable to the increase in energy expenditure (326). However, as all prior work has involved changes in energy expenditure (i.e. exercise *versus* no-exercise) a role of nutrient-exercise interactions independent of energy expenditure for PL remodelling is currently unclear. Nonetheless, as PGC1α activity may regulate changes to the PL content (327) and exercise with a reduced carbohydrate availability increasingly activates energy sensing proteins such as AMPK (311, 312), it could be speculated that exercising before *versus* after breakfast may stimulate PL remodeling to a greater extent via energy sensing (AMPK-PGC1α) pathways.

There is evidence to suggest that exercise training before *versus* after breakfast may increase oral glucose insulin sensitivity (**Figure 1.15**). However, this may not be the case when breakfast is completely omitted. If lean humans omit breakfast at rest over 6 weeks, interstitial glucose concentrations are more variable in the afternoon, relative to when breakfast is consumed (328). In humans with obesity, the insulinemic response to an OGTT is also lower with breakfast consumption *versus* omission over 6 weeks (329). As such, the effects of omitting breakfast but performing exercise training now warrants investigation. In addition, whilst nutrient-exercise interactions may regulate some adaptive responses to exercise in healthy men, this is yet to be investigated in humans with obesity.

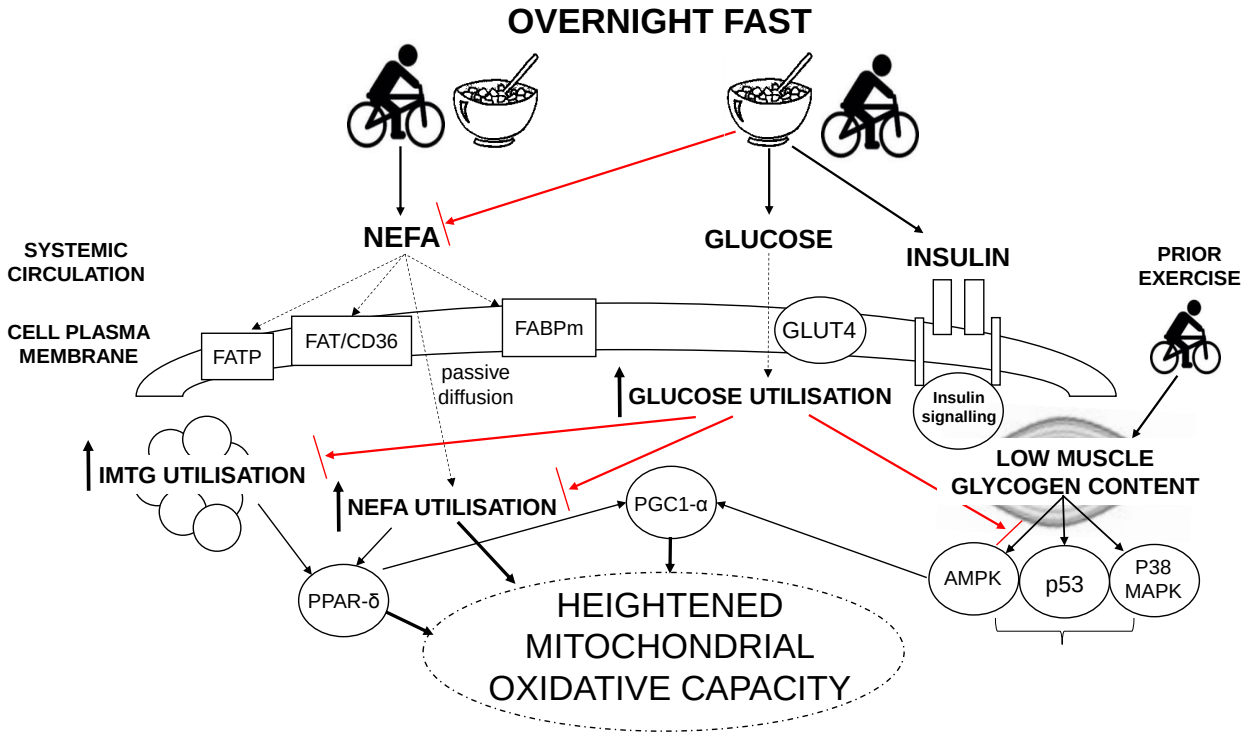


Figure 1.14. The effect of nutrient-exercise timing on mitochondrial adaptations following exercise training. After consuming carbohydrates, higher blood insulin and glucose concentrations decrease NEFA and IMTG utilisation during exercise. This may blunt the activation of adaptive signalling pathways and contribute, (along with the lower lipid flux during exercise) to the suppression of mitochondrial adaptations in skeletal muscle. In this figure red arrows show an inhibitory effect. Redrawn from a figure produced in (330).

Abbreviations:
GLUT4 = glucose transporter 4
FATP = fatty acid transport protein
FAT/CD36 = fatty acid translocase/cluster of differentiation 36
FABPm = fatty acid binding protein
NEFA = non-esterified fatty acid
IMTG = intramuscular triglyceride
AMPK = 5' AMP-activated protein kinase
P53 = tumour protein p53
P38 MAPK = P38 mitogen-activated protein kinases
PGC1α = peroxisome proliferator activated receptor gamma coactivator 1 alpha
PPARδ = peroxisome proliferator-activated receptor delta

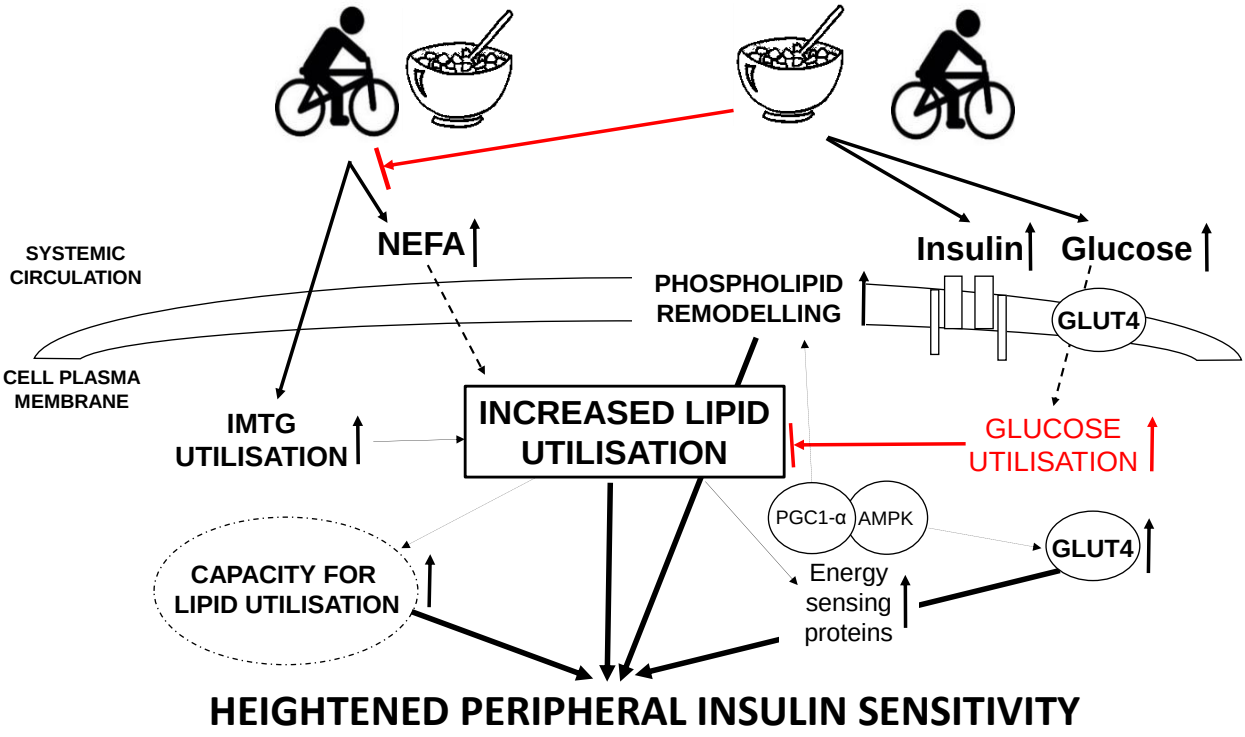


Figure 1.15. Potential pathways linking nutrient-exercise interactions to changes in peripheral insulin sensitivity. These include alterations in the exercise-induced increase in the accumulation (or activation) of key energy sensing proteins (e.g. AMPK), changes to the phospholipid content of plasma membranes, adaptations relating to the glucose transporter proteins and/or an increased capacity for lipid utilisation (see **Figure 1.14**). In this figure red arrows show an inhibitory effect.

Abbreviations:
NEFA = non-esterified fatty acid
IMTG = intramuscular triglyceride
AMPK = 5' AMP-activated protein kinase
PGC1α = peroxisome proliferator activated receptor gamma coactivator 1 alpha
GLUT4 = glucose transporter 4

BREAKFAST, EXERCISE AND ENERGY BALANCE

As energy cannot be created or destroyed (i.e. the first law of thermodynamics), energy balance can be calculated as metabolisable energy intake minus energy that is expended (151). As such, the long-term storage of excess energy, which manifests as obesity, must reflect either a sustained increase in energy intake, a sustained decrease in energy expenditure, or both when relative to a prior state of energy balance. Metabolisable energy intake represents the energy that is consumed in the diet in the form of carbohydrates (~ 4 $kcal \cdot g^{-1}$) fat (~ 9 $kcal \cdot g^{-1}$), protein (~ 4 $kcal \cdot g^{-1}$) and alcohol (~ 7 $kcal \cdot g^{-1}$) minus energy that is not absorbed. In humans, the energy that is not absorbed (lost in stools or urine) is around 5 % of ingested calories. Indeed, across a variety of diets it was shown that ~ 4.5 % and ~ 3.6 % of the energy ingested by healthy humans was lost in faeces or in urinary excretion, respectively (331). However, this percentage does depend on diet composition (332) and body composition, which may alter the gut microbiome and as a consequence, the energy harvesting capacity of the gastrointestinal tract (333). One further complication is that absorption of ingested energy can also be influenced by other factors, such as food preparation. For example, freezing and toasting bread increases the recrystallization of resistant starch reducing the fraction of the carbohydrate that is liberated into the systemic circulation and the ingestion of beef mince *versus* steak results in a higher availability of systemic amino acids (334, 335).

Daily energy expenditure is the summation of *(a)* resting metabolic rate (RMR; the energy required to maintain various cellular and tissue functions during rest such as fuelling sodium/potassium pumps, facilitating neuronal firing in the brain and maintaining core body temperature), *(b)* diet-induced thermogenesis (DIT; the increment in energy expenditure above the fasted-state required to process ingested food and drink) and *(c)* physical activity energy expenditure (PAEE; the energy attributable to the metabolic cost of exercise and non-exercise physical activity) (151). For the majority of people living in developed countries (e.g. non-athletes and people with jobs that require them to be predominantly sedentary), RMR represents about 60-65 % of daily energy expenditure [0.863 $kcal \cdot kg^{-1} \cdot h^{-1}$

(336)] and fat-free mass - specifically skeletal muscle metabolism - is the primary determinant of any inter-individual differences in RMR (337). DIT represents a smaller component of daily energy expenditure and is estimated to be ~ 10 % of total daily energy intake, although the diet composition can alter that percentage (338). Indeed, the energy required (as a percentage of energy ingested) to digest and absorb fats, carbohydrates, alcohol and protein is ~ 0-3 %, 5-10 %, 15-27 % and 20-30 %, respectively (338). The component of daily energy expenditure that shows the highest variation in most non-athletic cohorts is PAEE, with variance of > 1,300 kcal·day^{-1} (339). PAEE represents the energy necessary to exert physical forces on our environment and is primarily determined by the energy expended during activities of daily living, but when performed regularly, exercise can also make a contribution of > 20 % of average daily PAEE (340).

To facilitate body mass losses and to alleviate the presence of obesity, an energy deficit must be created and sustained by increasing energy expenditure and/or reducing energy intake (151). Whole-body fat mass losses at 0.5-1.0 kg·week^{-1} are thought to be sustainable and because the energy density of the adipose tissue is approximately 7.1 kcal·g^{-1}, to achieve this rate of fat mass loss an energy deficit of around 500 kcal·day^{-1} would be required (341).However, the magnitude of the deficit needed to incur a meaningful reduction in obesity prevalence is complicated by changes in body mass which can alter the energy cost associated with RMR. For example, an increasing prevalence of obesity at the population level can theoretically be explained by a positive energy balance of 7 kcal·day^{-1} (341). However, to have maintained this energy surplus at an individual level through increased energy intake alone (i.e. assuming no decrement in PAEE), a more substantial increase in energy intake of ~ 200 kcal·day^{-1} would theoretically be required to account for increases in energy expenditure attributed to an increase in RMR (341). The two sides of the energy balance equation are also regulated by various interlinking factors, which all contribute to a highly-evolved feedback system designed to preserve a state of energy balance (151). As such, any acute deviation away from a state of energy balance may alter subsequent energy intake or PAEE in an attempt to restore homeostasis. In particular, the

regulatory system(s) involved in a maintenance of energy balance are thought to be biased towards weight gain. For example, in response to a decrease in energy availability over 3 days, RMR and sympathetic activity decrease and muscular work becomes more efficient in an attempt to align energy expenditure to energy intake [which would be conducive to long-term survival via maintenance of energy balance (342)]. However, in that study, a week of overfeeding with a 50 % energy surplus did not result in a clear adaptive increase in energy expenditure.

Similarly, associations have been shown between an infrequent consumption of breakfast at rest (a *negative energy balance*) and risk of adiposity (a sustained *positive energy balance*), suggesting that compensation is likely to occur for the energy deficit created from the omission of breakfast (343, 344). It has since been shown that if healthy humans omit breakfast at rest they compensate through a higher energy intake at lunch (345). Although energy intake up to and including lunch remained higher with breakfast consumption *versus* omission (1246 ± 380 *versus* 929 ± 317 kcal, $p<0.01$) this difference of ~ 300 kcal was less than the 700 kcal provided in the breakfast. In healthy humans, consuming 700 kcal before 1100 over 6 weeks also results in a higher self-reported energy intake relative to when fasting is continued until midday (2730 ± 573 *versus* 2191 ± 494 kcal·d^{-1}, $p<0.01$) but the difference of ~ 540 kcal was again less than the energy provided in the breakfast (328). Similar results have been reported for humans with obesity (329). In these studies, the omission of breakfast also decreased morning PAEE and resulted in a lower 24-h PAEE in the healthy humans (328, 329). This could be important if a reduction in energy intake (e.g. with breakfast omission) was implemented as a weight loss strategy. For example, (in humans without obesity) a compensation for breakfast omission over a period of 24 h via a higher post-breakfast energy intake of ~ 150 kcal and a lower daily PAEE of ~ 450 kcal would create an energy deficit of only ~ 100 kcal relative to when a 700 kcal breakfast was consumed. As such, if this difference in energy balance was always apparent with the omission *versus* the consumption of breakfast, a specific decrease in fat mass would require a longer time period to occur, as the overall energy deficit is lower. Indeed, an energy deficit of ~100 kcal·day^{-1} would equate to a loss of fat

mass of only ~ 15 g·day^{-1} or ~ 0.1 kg·week^{-1} (341). Although even small changes to energy balance can alter fat mass over a sufficient period of time (346), this clearly highlights the important role of compensation for acute energy deficits in a context of long term changes in fat- and/or body-mass.

At the population level, associations exist between low physical activity levels and a higher risk of obesity (347). However for weight loss in the *initial months* of an intervention, exercise training can be less effective than energy restriction via diet (348). Changes in body-mass in response to exercise training also shows some apparent inter-individual variability [ranging from losses of 9.5 to gains of 2.6 kg after 12 weeks of exercise, designed to expend 2500 kcal·week^{-1} (349)] although this may be partly due to measurement errors or intra-individual variability (350). PAEE is suggested to be sustained if overweight men start to regularly exercise (340), so an increase in energy intake may be most accountable for this modest and variable weight loss response. The nutrient-status of participants prior to exercise is often unreported in exercise training studies and this may alter post-exercise compensation. Carbohydrate balance (dietary intake *versus* utilisation), is proposed to be important in this regard, as liver and muscle glycogen stores are thought to be tightly regulated, due to their small storage capacity (17, 351). As such, when these stores are utilised, behaviours that alter energy balance might change to favour their replenishment (17, 351). If carbohydrate balance is indeed a regulator of energy balance, reducing rates of carbohydrate utilisation during exercise may help prevent compensation for the exercise energy deficits.

When energy balance is manipulated with pre-exercise breakfast consumption *versus* omission in healthy men, no clear differences in energy intake at a mixed macronutrient lunch are reported (15). If breakfast (444 kcal; 60 % carbohydrate; 23 % fat; 17 % protein) was then accounted for, exercise and breakfast omission produced a more negative energy balance after the lunch compared to exercise and breakfast consumption. Similarly, if healthy men run for 60 min but without eating breakfast, *ad libitum* energy intake over 24 h is lower than when exercise is performed after eating breakfast (16). A lower energy intake with exercise

during morning fasting may be attributable to having less time in the day to eat. However in the second study, a lower energy intake with exercise and breakfast omission was partly attributable to a lower energy intake at post-exercise meals (16). This suggests that healthy men do not compensate with a higher energy intake for the energy deficit created by omitting breakfast prior to performing exercise, potentially facilitating a more negative energy balance relative to when breakfast is consumed. However, in the work of Bachman *et al.* (16) post-exercise energy-expenditure was not measured and so energy balance was not reported. A more negative energy balance after lunch with breakfast omission and exercise has also been attributed to a negative whole-body fat balance, with carbohydrate balance being similar to exercise with breakfast consumption (15). This is in line with a theory (discussed in **Chapter 5**), that carbohydrate stores in the body are tightly regulated (17, 352). Thus, sparing carbohydrate utilisation and inducing less severe fluctuations to carbohydrate balance with breakfast omission before exercise, may protect against energy compensation for the exercise-induced energy deficit. In contrast, an increased rate of carbohydrate use during exercise may increase the likelihood of a higher energy intake post-exercise (353, 354). More evidence that carbohydrate status (and specifically liver glycogen status) regulates energy balance is reports that mice overexpressing hepatic *protein targeted to glycogen*, (which increases glycogen synthesis by activating glycogen synthase and inhibiting glycogen phosphorylase), show a reduced energy intake, a higher energy expenditure and lower fat masses *versus* wild-type mice (355). Vagotomy (an operation where one or more branches of the vagus nerve are cut) also abolished these effects in mice without altering the liver glycogen content, suggesting a mediating role for the vagal nerve in co-ordinating any cross-talk between the liver and the brain regarding the carbohydrate status of the liver.

If increasing carbohydrate utilisation during exercise does stimulate an increased post-exercise energy intake, exercising before *versus* after breakfast (i.e. altering meal timing) may also be more effective for creating a negative energy balance. Regularly exercising before breakfast may also increase the capacity of skeletal muscle to utilise lipids, which may then reduce carbohydrate utilisation during

non-exercise activity (356). One study did report that if exercise was performed prior to breakfast, fat mass losses in 3 weeks were greater than if carbohydrates were consumed before- and during-exercise, despite the same nutrition being provided to both groups (357). During hyper-caloric high-fat feeding, exercise training before and not after breakfast may protect against body mass gains when compared to a non-exercise (control) group (11). However, changes in body composition do not typically differ after exercise training performed before *versus* after breakfast (12, 358, 359), although it should be noted that the length of the training in many of these studies (~ 4-6 weeks) may be insufficient to allow the detection of small differences in body composition across exercise groups. As there is incomplete compensation in energy intake for an omission of breakfast before exercise, this model (i.e. breakfast omission and exercise and not altered breakfast timing) is most likely to create the conditions for a more negative energy balance (and larger decreases in body mass over time) compared to exercise and breakfast consumption. Studying energy balance with pre-exercise breakfast omission *versus* consumption over 24 h (and then longer) is now important.

CONCLUDING STATEMENTS

Obesity is often accompanied by decreased peripheral insulin sensitivity, which is likely to be partly explained by defects relating to lipid metabolism in skeletal muscle. Exercise training improves many aspects of metabolic health, but finding ways to maximise these effects is important. Regularly exercising before *versus* after nutrient-intake can augment adaptations in skeletal muscle that might be important for both lipid metabolism and insulin sensitivity. Each bout of exercise before *versus* after nutrient-intake also increases lipid flux during the activity and this acute response may be important for any changes in insulin sensitivity with regular training. Finally, omitting *versus* consuming breakfast prior to exercise might create a more negative daily energy balance, which may have implications for the long-term regulation of body mass if this stimulus is regularly repeated. However, the metabolic and energy balance responses to exercise before *versus* after nutrient-ingestion in humans with obesity has never been established.

GENERAL AIMS OF THIS THESIS

Accordingly, the general aims of this thesis were:

1) To explore the effects of nutrient-exercise interactions on substrate metabolism during and post-exercise.
2) To explore the effects of nutrient-exercise interactions on the regulation of post-exercise postprandial glucose metabolism and potential underlying regulatory mechanisms in healthy men.
3) To investigate the effects of nutrient-exercise interactions on intramuscular signalling responses in healthy men.
4) To explore the effects of nutrient-exercise interactions on training responsiveness in humans classified as overweight and obese, with a specific focus on glucose metabolism and oral glucose insulin sensitivity.
5) To explore the effects of nutrient-exercise interactions during exercise training in humans classified as overweight or obese on skeletal muscle adaptations.
6) To assess the effects of nutrient-exercise interactions on energy and substrate balances over a period of 24 h.
7) To assess the effects of nutrient-exercise interactions for changes in body mass and components of human behaviour that regulate energy balance with exercise training in humans classified as overweight or obese.

CHAPTER 2 - GENERAL METHODS

This chapter describes the experimental methods that were used consistently throughout this thesis. The studies received ethical approval from the Research Ethics Approval Committee for Health (REACH) for the University of Bath and/or the National Health Service (South West) Research Ethics Committee. Written, informed consent was gained from participants prior to them embarking upon any given study. All studies were undertaken at the University of Bath in accordance with the Declaration of Helsinki.

PARTICIPANTS

Participants were primarily recruited from the staff and student population at the University of Bath or the surrounding area (i.e. Bath and North East Somerset) by email, posters or word of mouth. After contacting the research team to express an interest in a particular study, prospective participants were provided written information about the requirements of the study and the benefits and risks of taking part and were given opportunities to ask any questions they had. If they decided to take part, the participants completed a signed statement of informed consent form and a health history questionnaire to confirm their eligibility for the study and to help ensure their safety and wellbeing throughout their involvement. In **Chapters 3-5**, participants were mainly recruited from the University of Bath and were healthy, physically active men. The inclusion criteria were:

- Males, aged 18-49 years
- Recreationally active
- Non-smokers
- Able and willing to safely comply with all study procedures
- Able to provide written informed consent for participation
- No bleeding disorder or taking medication which impacts blood coagulation
- No use of substances which may have posed undue risk to the participant or have introduced bias into the experiment

• No contraindications to exercise testing, as determined by using a readiness for exercise questionnaire (i.e. the Physical Activity Readiness Questionnaire)
• No known history of metabolic or cardiovascular disease
• Not currently dieting and having been weight-stable for the preceding 3 months (< 5 % change in body mass)
• No plans to change diet or physical activity behaviours outside of the study
• No aversion to any of the foods provided in the study
• Additional inclusion criteria were also included for the participants who opted to have muscle biopsy samples (for the research described in **Chapters 3 and 6**). These were; no known tendency towards keloid scarring and no known sensitivity or allergy to any local anaesthetic medicines

In **Chapter 6** participants were recruited from the staff and student population at the University of Bath or the local surrounding area and were classified as overweight or obese and sedentary. The inclusion criteria (where differing from above) were:

• Males, aged 18-65 years
• Classified as overweight according to the body mass index (BMI) > 25 $kg \cdot m^{-2}$
• Self-reported non-exercisers when exercise was defined to them as *'continuous physical activity lasting longer than 30 mins and of sufficient intensity for you to perceive a notable increase in your heart rate and/or a sweat response'*

ANTHROPOMETRY

For anthropometric data, measurements were made in duplicate and averaged, but if these deviated by > 1 %, a third measure was also recorded. Stature was measured to the nearest 0.1 cm using a wall-mounted stadiometer (Seca Ltd, Birmingham, UK), with participants barefoot and in the Frankfurt plane and after inhalation of a breath. Body mass was measured with participants wearing light clothing only, to the nearest 0.1 kg using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). The body mass index (BMI) was calculated as body mass (kg) divided by the square of the participant's height (m). Waist and

hip circumference were assessed to the nearest 0.1 cm using a non-elastic tape measure (SECA 201, Hamburg, Germany) and the waist-to-hip circumference was calculated. Waist circumference was taken at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest and hip circumference was measured at the widest part of the buttocks. For **Chapters 3 and 5,** a dual energy x-ray absorptiometry (DEXA) scan was completed to quantify fat- and fat free-mass (Discovery, Hologic, Bedford, UK). The principle of DEXA is that the distribution of bone, fat and non-bone or -fat tissues can be estimated from pixels on a planar image, according to their propensity for radiographic attenuation at two frequencies (360). This approach has some limitations for assessing body composition, for example, fat-free mass estimations are susceptible to changes in hydration status and the scan gives a cross-sectional image in the frontal plane and so does not account for trunk thickness in the sagittal plane, which might underestimate central adiposity. However, it provides data that cannot be derived from more simple measures of body composition. To improve scan accuracy, quality control checks were completed before each DEXA scan, which included scanning a spine phantom with known radiographic attenuation properties and checks to monitor background radiation. Scans were performed with participants wearing light clothing, having adhered to the pre-trial controls as detailed below, and having consumed 568 mL of water 1 h prior and voided immediately prior, to help control for hydration status. They were positioned centrally on the scanning bed in a supine position, with legs apart, feet turned inwards, arms as wide as possible and hands in a mid-prone position. This was to ensure that the gap between their arms and trunk was sufficient to allow regions of interest to be defined on the analysis software (QDR for Windows, Hologic, UK). Scans were analysed for fat- and fat-free mass to the nearest 0.1 kg, percentage body fat and fat mass index (fat mass [kg] divided by the square of the participant's height [m]).

STANDARDISATION PROTOCOLS

For laboratory-based trials, the ambient temperature, barometric pressure and humidity were assessed using a portable weather station (Technoline WS 6730, TechnoTrade Import-Export GmbH, Berlin, Germany). These conditions were

controlled so that for each participant all trials were performed in similar ambient conditions (details are provided in each experimental chapter). For all laboratory-based trials participants were asked to abstain from alcoholic and caffeinated drinks for 24 h prior to testing. Food intake ceased at 8 pm on the evening prior to each trial and participants fasted overnight for a minimum of 12 h, consuming only water *ab libitum* during this period. They recorded the composition of their evening meal on the day before the first main trial for a given study and replicated the meal for subsequent trials in accordance with procedures for standardising glucose tolerance testing (361). Participants were asked to refrain from strenuous physical activity for 24 h prior to trials but to otherwise maintain their habitual lifestyle. Weighed food and fluid records and/or questioning by a researcher assessed their compliance to these controls. In **Chapters 3 and 5**, participants also completed a physical activity diary and were given a physical activity monitor (ActiheartTM; Cambridge Neurotechnology, Papworth, UK) to wear for the day before each trial, as reported in the chapters. Water consumption was *ad libitum* on the first trial for a given study and was replicated for subsequent trials.

EXPIRED GAS SAMPLES

Estimating rates of substrate utilisation during rest and exercise was pertinent to the aims of this thesis and was assessed using indirect calorimetry from expired gas samples. The respiratory quotient (RQ) is the quantity of CO_2 produced *versus* O_2 consumed at the tissue level. Indirect calorimetry assesses whole body O_2 consumption and CO_2 production by using measures of gas exchange at the lungs, as an approximate measure of the RQ. This indirect measure of the RQ is referred to as the respiratory exchange ratio (RER). At the whole-body level, the ratio of CO_2 produced *versus* O_2 consumed depends on the ratio of substrates being oxidised, because the RQ of glucose is 1.00 (6 mol of O_2 are consumed and 6 mol of CO_2 are produced for each mole [180 g] oxidised) and for palmitoyl-stearoyl-oleoyl-glycerol (PSOG), a triglyceride that is similar in composition to the composition of human adipose tissue, the RQ is 0.70 (78 mol of O_2 are consumed and 55 mol of CO_2 produced for each mole [861 g] oxidised) (88). It is possible to measure an RER of > 1.00 using indirect calorimetry under conditions of a

sustained positive energy (particularly carbohydrate) balance (27, 362), because of net *de novo* lipogenesis, where lipid production is exceeding lipid oxidation at the whole-body level. This is because the process of *de novo* lipogenesis has an RQ of ~ 5.6, due to a net consumption of oxidising equivalents (88). Substrate utilisation represents the sum of oxidation and any endogenous interchange of substrates, such as *de novo* lipogenesis. Whilst humans have the capacity to convert carbohydrates and proteins into lipids, we lack the enzymes necessary for the reverse, so fatty acids can only be oxidised or stored, meaning that substrate utilisation is equivalent to oxidation. However, for carbohydrates, this is not always the case, so in this thesis the term substrate utilisation has been used. However, in the conditions studied (the absence of a sustained positive energy or carbohydrate balance) carbohydrate utilisation can be interpreted as oxidation.

Continuous breath-by-breath gas analysis

Incremental cycling tests were undertaken to assess substrate utilisation rates, peak oxygen uptake ($\dot{V}O_2$ peak) and peak power output (PPO). The protocols were adapted from previous research that assessed substrate utilisation rates across different exercise intensities and durations (363). Tests were performed at a self-selected cadence using an electronically-braked ergometer (Excalibur Sport, Lode® Groningen, Netherlands). Participants were allowed to adjust the saddle and handlebar heights to a preferred position, which were replicated for any further cycling in a given study. For **Chapters 3 and 4**, the initial power output was set at 50 Watts (W) and this was increased by 50 W every four min for an initial four stages. Thereafter, the intensity was increased by 20 W every min until volitional exhaustion. A two-phase protocol was chosen to increase the accuracy of the test when assessing substrate utilisation during the initial stages as indirect calorimetry requires steady state expired O_2 and CO_2 concentrations, and then to facilitate the attainment of volitional exhaustion quickly, to reduce the likelihood that localised muscle fatigue was the reason for test termination. Heart rate (HR; Polar Electro Oy, Kempele, Finland), ratings of perceived exertion [RPE; from 6 (no exertion) to 20 (maximal exertion) (364)] and continuous breath-by-breath measurements were recorded throughout tests. The breath-by-breath analyser

(TrueOne2400, ParvoMedics, Sandy, USA) was turned on 60 min prior to testing to allow for the temperature of the O_2 and CO_2 sensors to stabilise. The volume and gas analysers were calibrated with a 3 L calibration syringe (Hans Rudolph, Kansas City, USA) and a known concentration of a calibration gas (16.04 % O_2, 5.06 % CO_2; BOC Industrial Gases, Linde AG, Munich, Germany). During tests participants breathed through a mouthpiece that was connected to a two-way, non-rebreathing valve (model 2730, Hans Rudolph, Kansas City, Missouri), which was itself connected to the analyser via falconia tubing (Baxter, Woodhouse & Taylor Ltd, Macclesfield). After the test, PPO was calculated as the work rate of the last completed stage, plus the fraction of time in the final non-completed stage, multiplied by the relevant W increment. Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were averaged over the last min of each stage and used to assess substrate utilisation rates. $\dot{V}O_2$ peak was calculated as the highest average $\dot{V}O_2$ measured over a rolling 30 s period. At least two criteria needed to be achieved for a valid $\dot{V}O_2$ peak measure; a HR < 10 beats·min^{-1} of age predicted maximal (220 - age), a respiratory exchange ratio (RER) > 1.05, an RPE > 17 or an increase in $\dot{V}O_2$ of < 5 ml·kg^{-1}·min^{-1} despite further increments in the exercise intensity (365). In **Chapter 6**, the protocol was slightly modified to account for an expected lower fitness level of individuals not accustomed to exercising, whereby the test started at 50 W and the work rate increased by 25 W every three min.

Douglas bag analysis

During rest and exercise, with the exception of the exercise tests, Douglas bags were used to collect samples of expired air. The sensor (Mini MP 5200, Servomex Group Ltd., Crowborough, UK) was turned on 60 min prior to testing to allow for the temperature of the O_2 and CO_2 sensors to stabilise and was then calibrated using a two-point calibration (99.99 % Nitrogen, 0 % O_2, 0% CO_2 and 16.04 % O_2, 5.06 % CO_2 calibration gases: BOC Industrial Gases, Linde AG, Munich, Germany). Participants were provided with a mouthpiece one min before each sample was collected as a stabilisation period. Expired air samples were collected into 200 L Douglas bags (Hans Rudolph, Kansas City, USA) with the mouthpiece connected to a two-way, T shaped non-rebreathing valve via falconia

tubing and measurements of ambient O_2 and CO_2 concentrations were recorded (366). Expired O_2 and CO_2 concentrations were measured in a known volume of each sample for two min, or longer if values were not stable, using paramagnetic and infrared transducers in the sensor. The ambient temperature and barometric pressure were recorded as detailed previously, so that expired air volumes could be corrected to standard temperature and pressure for dry gases. The volume and temperature of expired air samples were measured using a dry gas meter (Harvard Apparatus) and a digital thermistor (HI98509 Checktemp 1, Hanna Instruments Ltd, Bedford, UK), respectively. The Haldane transformation was used to calculate inspired gas volumes and to determine $\dot{V}O_2$ and $\dot{V}CO_2$ (367).

Rates of lipid and carbohydrate utilisation were estimated from $\dot{V}O_2$ and $\dot{V}CO_2$ values via stoichiometric equations, primarily based on the formulae reported by Keith Frayn (88). The equation used for lipids was based on the oxidation of palmytoyl-stearoyl-oleoyl glycerol [PSOG], because this triglyceride is similar to the overall composition of human adipose tissue. For carbohydrate utilisation, calculations were also based on work by Frayn (88) but adjustments were made to account for glycogen utilisation during exercise. Specifically, it was assumed that there was an equal contribution of glucose and glycogen to carbohydrate utilisation at 40-50 % $\dot{V}O_2$ peak, but with 20 % and 80 % contributions respectively at exercise intensities of > 50 % $\dot{V}O_2$ peak (67). The underlying calculations are explained subsequently in this chapter and the equations used were:

1) Lipid utilisation for rest and exercise ($g \cdot min^{-1}$): $1.67 \times \dot{V}O_2 - 1.67 \times \dot{V}CO_2$
2) CHO utilisation for rest ($g \cdot min^{-1}$): $4.542 \times \dot{V}CO_2 - 3.201 \times \dot{V}O_2$
3) CHO utilisation for exercise at 40-50 % $\dot{V}O_2$peak ($g \cdot min^{-1}$):

$$4.344 \times \dot{V}CO_2 - 3.061 \times \dot{V}O_2$$

4) CHO utilisation for exercise at 50-75 % $\dot{V}O_2$ peak ($g \cdot min^{-1}$):

$$4.210 \times \dot{V}CO_2 - 2.962 \times \dot{V}O_2$$

Substrate utilisation rates were only determined for exercise at intensities below 75 % $\dot{V}O_2$ peak, due to increasingly inaccurate estimations of substrate utilisation

from indirect calorimetry at higher exercise intensities (67). Energy expenditure was calculated assuming that lipids, glucose and glycogen yield 9.42, 3.74 and 4.15 kcal·g^{-1} of energy, respectively (67). The precise mixture of lipid sources utilised is generally unknown, so caloric equivalents from 6.8 to 9.8 kcal·g^{-1} have been previously cited (67). However, the most commonly applied constants are from 9-9.75 kcal·g^{-1} which is within 3-4 % of the value of 9.42 kcal·g^{-1} used here.

In **Chapters 3 and 5**, protein utilisation was accounted for in the measures of carbohydrate and lipid utilisation (88, 368). Before protein is utilised for energy it is first catabolised into amino acids and deaminated. The resultant amino acid can be utilised, but nitrogen and sulphur are excreted in urine, sweat and faeces. To measure protein utilisation, urinary nitrogen excretion was first estimated from urine urea concentrations. The total volume of urine produced during a given trial was recorded and a representative 1 mL of this sample was stored at -80 ºC. Urine urea concentrations in mmol·L^{-1} were measured as detailed subsequently. The concentration was divided by 35,700 to yield a urea concentration in g·mL^{-1} and then multiplied by 0.47 to yield nitrogen in g·mL^{-1}. This assumes nitrogen comprises 47 % of urea, but this is an estimate as urea accounts for ~ 90 % of the nitrogen excreted in urine. This value was multiplied by the total urine volume (mL) to estimate urinary nitrogen losses in g. Although, different values are used to calculate protein utilisation in g·min^{-1} from urinary nitrogen losses in g·min^{-1}, the commonly applied value of 6.25 g of protein per g of urinary nitrogen was applied here, as nitrogen comprises ~ 16 % of protein (67).

In **Chapters 4 and 6**, protein utilisation was assumed to have made a negligible contribution to energy metabolism for within-laboratory testing. Trial days in those studies were shorter (~ 3 h) and protein utilisation was unlikely to have made a meaningful contribution to metabolism as trials were mostly resting, meals were carbohydrate-based and any exercise was moderate-intensity and relatively short duration. During rest protein utilisation is ~ 1.4 g·h^{-1} for healthy men making it a small contributor to energy metabolism (369). In a bolus, considerable doses of protein (> 40 g) need to be ingested to stimulate protein utilisation above these

values (370) and even high-intensity exercise does not always stimulate protein utilisation above rest (369). As such, the absence of any correction for protein utilisation is unlikely to have meaningfully altered estimations of whole-body lipid or carbohydrate utilisation when it was not measured. In addition, for accurate measures of protein utilisation using nitrogen excretion, trial days should be of sufficient length for the sample to be reflective of daily nitrogen excretion (371).

GLUCOSE TOLERANCE AND INSULIN SENSITIVITY

Although variations exist, some methods are most commonly used for assessing glucose tolerance or peripheral insulin sensitivity (372). These involve measuring blood glucose and insulin concentrations during fasting or after a physiological challenge such as an oral glucose tolerance test. Peripheral insulin sensitivity can be directly assessed with a hyperinsulinaemic-euglycemic clamp, whereby insulin is infused at a constant rate to maintain hyperinsulinemia and blood glucose is maintained at around 5.0 $mmol \cdot L^{-1}$ using a second infusion, with a higher glucose infusion rate indicating heightened peripheral insulin sensitivity. Although less common, a hyperinsulinaemic-hyperglycaemic clamp can also be used, whereby glucose concentrations are increased to induce hyperglycaemia and maintained through an infusion, with a higher infusion rate indicating high peripheral insulin sensitivity (372). However, the gold-standard method for assessing whole-body glucose tolerance is the oral glucose tolerance test (OGTT), where a 75 g glucose bolus is ingested and followed by blood sampling every 10-15 min for ~ 120 min. After 120 min, blood glucose concentrations of 7.8 to 11.0 $mmol \cdot L^{-1}$ suggests impaired glucose tolerance and concentrations of > 11.0 $mmol \cdot L^{-1}$ suggests T2D (372). In this test, the rate of blood glucose appearance is dependent on gastric emptying, intestinal absorption and liver function, so it is more representative of free-living situations (meal ingestion) than infusion-based methodologies (372). For example, gastro-intestinally derived hormones such as GLP-1 are secreted from intestinal enteroendocrine cells after meal consumption and can potentiate insulin secretion (40), but this would not be apparent when glucose or insulin are only infused peripherally. Therefore, the OGTT mimics the glucose and insulin dynamics of physiological conditions more closely than infusion based methods.

In addition, many indices of insulin sensitivity have been developed and validated against a hyperinsulinaemic-euglycemic clamp, using measures obtained from an OGTT. These indices include, but are not limited to, the homoeostatic model assessment of insulin resistance [HOMA2-IR; fasting plasma glucose ($mmol \cdot L^{-1}$) x fasting plasma insulin ($pmol \cdot L^{-1}$) / 22.5 (373)], the Quantitative Insulin Sensitivity Check Index [QUICKI; 1 / log fasting blood insulin ($\mu U \cdot ml^{-1}$) + log fasting blood glucose ($mg \cdot dL^{-1}$) (374)], the Matsuda Insulin Sensitivity Index ($ISI_{MATSUDA}$; 10,000 / √ fasting blood glucose ($mg \cdot dL^{-1}$) x fasting blood insulin ($mIU \cdot mL^{-1}$) x mean glucose over 120 min ($mg \cdot dL^{-1}$) x mean insulin over 120 min ($mIU \cdot mL^{-1}$) (296)], the Cederholm Insulin Sensitivity Index *(*$ISI_{CEDERHOLM}$; 75000 + fasting blood glucose ($mmol \cdot L^{-1}$) - blood glucose at 120 min ($mmol \cdot L^{-1}$) x 0.19 x 180 x 1.15 x body mass / 120 x log mean blood insulin over 120 min ($mIU \cdot mL^{-1}$) x mean blood glucose over 120 min ($mmol \cdot L^{-1}$) (375)] and the oral glucose insulin sensitivity index [OGIS; calculated as per http://webmet.pd.cnr.it/ogis/ (376)].

In contrast to some OGTT-derived insulin sensitivity indices, such as $ISI_{MATSUDA}$, which are based on more simple empirical formulas, the OGIS was developed as a physiologically representative glucose-insulin model. This method provides an index of insulin sensitivity calculated via a model-derived formula from the OGTT glucose and insulin concentrations from 0 min, 90 min and 120 min. Specifically, a single compartment glucose model is used to estimate blood glucose clearance rates after accounting for body surface area, similar to the hyperinsulinemic-euglycemic clamp. The index was validated against the clamp using 104 humans with and without obesity and T2D. The authors reported that (*a*) the OGIS showed a stronger correlation with the clamp method than other commonly-used insulin sensitivity indices (*b*) there was no mean difference between the glucose clearance rates derived from the clamp and the OGIS index and that (*c*) the OGIS showed good intra-individual reproducibility. However, the index has only been validated in resting and fasting conditions and thus if the mechanisms governing the glucose-insulin relationship and the modelling from which the index was derived differ to those conditions, the OGIS should be used more cautiously.

In this thesis, fasting and OGTT-derived blood glucose and insulin concentrations were measured. An OGTT was chosen to permit a simultaneous assessment of whole-body oral glucose tolerance and an estimation of oral glucose insulin sensitivity and because it was feasible given other constraints within each study. Although the OGTT has been commonly used in physiology research, different methods are used to obtain blood samples and include sampling from antecubital veins (15), capillaries (377) or heated dorsal hand veins (329). Arterial blood best represents the exposure of the peripheral tissues to metabolites and hormones in the blood, so when knowledge of peripheral exposure to glucose or insulin is important, arterial blood samples are appropriate. However, due to the increased risks associated with arterial catheterisation (378) other methods have been used to provide samples reflective of arterial blood, such as the heated-hand technique (379-385), which was used throughout this thesis.

BLOOD SAMPLING AND TREATMENT

Upon arrival at the laboratory and after 10-15 min of rest, participants placed their dominant hand into a heated-air box set to 55°C (Mass Spectrometry Facility; The University of Vermont & University of Vermont Medical Center, Burlington, USA). After 10-15 min, an intravenous catheter (BD Venflon Pro, BD, Helsingborg, Sweden) was placed retrograde into a heated dorsal hand vein and a 10 mL baseline blood sample was drawn. Blood was then sampled according to the design of each study. Cannulae were kept patent via flushing of a 0.9 % sodium chloride infusion (B.Braun, Melsungen, Germany) and the first 3 mL of drawn samples was discarded to avoid any confounding influence of saline dilution on the measured concentrations of metabolites or hormones in the sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and first frozen at -20°C, before storage at -80°C.

Automatic analyser (Daytona)

Various metabolites were measured in plasma using an automated analyser (Daytona; Randox Lab, Crumlin, UK). Before any analysis, the analyser was calibrated using a two-point linear calibration system with a blank and a sample with a known concentration of the metabolite. After calibration and at frequent intervals during the analysis, standard samples were measured to act as a quality control check. Before analysis, plasma samples were removed from the -80 ºC freezer and thawed on ice for 60 min before measurement. Plasma glucose concentrations were assessed in a colorimetric assay that measures glucose after enzymatic oxidation by glucose oxidase, which produces gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with phenol and 4-aminophenazone in a reaction catalysed by peroxidase to produce a red-violet quinoneimine dye. The intensity of the colour produced is measured at 505 nm and is proportional to the sample glucose concentration up to 34.1 $mmol \cdot L^{-1}$. For plasma lactate concentrations up to 15.0 $mmol \cdot L^{-1}$, lactate in the sample was converted to pyruvate and hydrogen peroxide by lactate oxidase, before the same hydrogen peroxide reaction as per the measurement of glucose.

Plasma triglyceride (TAG) concentrations were measured using a colorimetric assay, whereby enzymatic hydrolysis of TAG by lipases forms glycerol and fatty acids. The glycerol is then converted to glycerol-3-phosphate by glycerol kinase and glycerol-3-phosphate is then oxidised by glycerol-3-phosphate oxidase to form dihydroxyacetone and hydrogen peroxide. Finally, quinoneimine is formed from the hydrogen peroxide, 3-aminophenzane and 4-cholorophenol in a reaction catalysed by peroxidase and is measured at 505 nm up to TAG concentrations of 12.7 $mmol \cdot L^{-1}$. However, as this assay measures the glycerol component of TAG it can overestimate TAG concentrations if substantial glycerol is present in the sample. To avoid this potential measurement issue, glycerol concentrations were independently measured on the analyser to provide a glycerol-blanked TAG concentration. Concentrations of *β*-hydroxybuturate were assessed in an assay that oxidises *β*-hydroxybuturate to acetoacetate in a reaction that is catalysed by *β*-hydroxybuturate dehydrogenase. In this reaction NAD^+ is reduced to form

NADH and the resultant change in absorbance is measured and correlated with the *β*-hydroxybuturate in a sample up to concentrations of 0.10-5.75 mmol·L^{-1}. Finally, to allow for protein utilisation to be estimated, urine urea concentrations were measured. In this assay, urea is hydrolysed by urease to produce ammonia and CO_2, before the ammonia then combines with α-oxoglutarate and NADH in a reaction catalysed by glutamate dehydrogenase to form glutamate and NAD^+. A change in absorbance can then be measured and correlated with the urea concentration in the sample.

Enzymatic colorimetric assays

Plasma non-esterified fatty acid (NEFA) concentrations were determined via a commercially available assay using an enzymatic colorimetric principle (Alpha Laboratories Ltd; Hampshire, UK). Briefly, 5 µL of each sample was pipetted into a well and incubated for 5 min at 37 °C in the presence of 200 µL of an Acyl-CoA synthetase, coenzyme A and adenosine 5-triphosphate disodium salt mixture. This converts NEFA in the sample to acyl-CoA, adenosine monophosphate and pyrophosphoric acid (PPi). The absorbance at 550 nm was measured to serve as a blank (SPECTROstar plate reader, BMG Labtech, Aylesbury, UK). Then, a reagent containing acyl-CoA oxidase and peroxidase was added before a 5 min incubation. This was to oxidise the acyl-CoA to yield 2 3-trans-Enoyl-CoA and hydrogen peroxide. Finally, in a reaction catalysed by peroxidase, the hydrogen peroxide yields a blue pigment after oxidation condensation with 3-Methyl-N-Ethyl-N (*β*-Hydroxyethyl)-Aniline and 4-amino-antipyrine. The absorbance was read again at 550 nm and the reading was subtracted from the blank for a final absorbance. The concentration of NEFA in each sample was determined using the relationship between standard concentrations and the final absorbance.

Enzyme-linked immunoassays (ELISA)

Enzyme-linked immunosorbent assay kits (ELISAs) were used to measure insulin, leptin (both Mercodia AB; Uppsala, Sweden), fibroblast growth factor 21 (FGF21; BioVendor Research & Diagnostic Products Czech Republic) and intestinal fatty acid binding protein (IFAB-P; Cambridge Bioscience Ltd; UK)

concentrations using the direct sandwich technique. For insulin, 25 µL of plasma was added to wells coated with a monoclonal antibody directed towards the antigenic site on insulin. A second monoclonal antibody was added which was labelled with horseradish peroxidase (HRP), which provides the enzyme activity to convert the substrate to a fluorescent product. The mixture was left for 60 min at room temperature on a plate shaker at 900 rpm. After washing to remove any unbound antibody, tetramethyl benzidine (TMB) was added in a 200 µL solution and left for 15 min to start the HRP reaction. The reaction was stopped by adding a 50 µL sulfuric acid mixture and the absorbance was measured at 450 nm with the aforementioned plate reader. The concentration of insulin in each sample was determined with the relationship between insulin concentrations of standards and absorbance values for concentrations up to 200 $mU \cdot L^{-1}$.

To measure leptin a similar assay was performed, but antibodies were directed against antigenic sites specific to leptin. During an initial incubation leptin in the sample reacted with peroxidase-conjugated anti-leptin antibodies and after washing, bound conjugate was detected using the TMB reaction, before reading the absorbance at 450 nm for concentrations up to 100,000 $ng \cdot mL^{-1}$.

For I-FABP concentrations, 100 µL of plasma was pipetted into wells coated with I-FABP antibodies. After a 60 min incubation at room temperature, 100 µL of biotinylated tracer antibody was then added, which bound to captured I-FABP. After a 60 min incubation, 100 µL streptavidin-peroxidase was added and after a third 60 min incubation, the TMB reaction was initiated, before being terminated 30 min later with oxalic acid. The absorbance was read at 450 nm and I-FABP concentrations in each sample were assessed using the absorbance values and a standard curve for concentrations from 47-3,000 $pg \cdot mL^{-1}$.

FGF-21 concentrations were measured by adding plasma to wells coated with a polyclonal FGF-21 antibody. Following a 60 min incubation, 100 µL of a biotin-labelled polyclonal FGF-21 antibody was added and incubated for 60 min. Then, and similar to the measurement of I-FABP, streptavidin-peroxidase and TMB

were added. In this assay the TMB reaction was stopped using sulphuric acid before absorbances were read at 450 nm and 630 nm (readings at 630 nm were subtracted from those at 450 nm) to detect concentrations up to 1920 $pg \cdot mL^{-1}$.

Precision of analysis

To minimise inter-assay variation, all samples were analysed in batch against the same standards and controls after all data collection was completed. For blood analysis, all samples for a participant were measured on the same plate or same run on the Daytona. If concentrations were outside of the standard curve for a given assay, they were diluted as per the kit instructions and then re-analysed. Samples were measured in duplicate unless otherwise stated. Inter- and intra-assay variations are shown in **Table 2.1**.

2347

Table 2.1. Coefficient of variations for biochemical analytical techniques

	Method Used	Coefficient of Variation (%)	
		Intra-assay	Inter-assay
Glucose	**Photometric (Daytona)**		
Chapters 3 and 5		3.2 ± 1.6	3.8 ± 2.8
Chapter 4		2.9 ± 1.3	5.9 ± 1.3
Chapter 6		2.5 ± 1.2	3.8 ± 3.5
Triglycerides	**Photometric (Daytona)**		
Chapter 3		1.4 ± 0.2	4.0 ± 0.3
Chapter 4		3.4 ± 1.1	4.0 ± 1.1
Chapter 6		3.4 ± 1.2	4.1 ± 3.7
Lactate	**Photometric (Daytona)**		
Chapter 3		1.0 ± 0.1	4.8 ± 1.7
Chapter 4		3.4 ± 1.2	3.7 ± 0.6
Total Cholesterol	**Photometric (Daytona)**		
Chapter 6		1.2 ± 1.0	4.3 ± 4.1
HDL Cholesterol	**Photometric (Daytona)**		
Chapter 6		2.6 ± 3.4	3.9 ± 0.8
LDL Cholesterol	**Photometric (Daytona)**		
Chapter 6		0.8 ± 0.5	5.0 ± 4.9
Glycerol	**Photometric (Daytona)**		
Chapter 6		1.7 ± 0.0	4.7 ± 4.0
Insulin	**ELISA**		
Chapter 3		5.9 ± 2.2	10.0 ± 3.2
Chapter 4		3.7 ± 0.8	6.5 ± 2.9
Chapter 6		3.9 ± 1.3	9.9 ± 3.5
NEFA	**ELISA**		
Chapter 3		8.9 ± 3.3	10.5 ± 3.2
Chapter 6		7.9 ± 1.2	10.2 ± 2.9
IFAB-P	**ELISA**		
Chapter 3		6.0 ± 2.2	-
FGF-21	**ELISA**		
Chapter 5		-	-
Leptin	**ELISA**		
Chapter 5		5.8 ± 4.8	7.1 ± 5.0
C-peptide	**ELISA**		
Chapter 6		4.3 ± 1.8	17.8 ± 3.2

HDL= High density lipoprotein; LDL= Low density lipoprotein; NEFA= Non-esterified fatty acids; IFAB-P= Intestinal fatty acid binding protein; FGF-21= Fibroblast growth factor 21.

BLOOD GLUCOSE FLUX

To provide data on blood glucose flux during- and post-exercise, glucose tracers ([U^{13}-C]-glucose ingestion with an infusion of [6,-6- $^{2}H_2$]-glucose) were utilised in **Chapters 3 and 5**. The principles of this method are discussed elsewhere (29) but some important considerations are outlined below. Isotopes are atoms that are chemically identical but have different molecular weights due to a different number of neutrons in their nucleus. The common isotopes of major elements [hydrogen (H), carbon (C), nitrogen (N) and oxygen (O)], are ^{1}H, ^{12}C, ^{12}N and ^{16}O, and these are stable and non-radioactive. However, there are other stable isotopes, that can be administered safely to humans, which are less common (e.g. ^{2}H, ^{13}C, ^{15}N, and ^{18}O). These have the same chemical properties as the more common isotopes but can be distinquished with mass spectrometery, due to differences in their mass to charge ratio. For example, each glucose molecule has six carbons and each carbon atom has ~ 1.1 % of chance of being ^{13}C, so only 6.6 % of naturally occurring glucose has one ^{13}C. The underlying principle of tracer methods is that when a known amount of a tracer is added to a system, sampling from the pool (e.g. plasma) where the tracer is mixed to measure tracer dilution, provides information about the flux of a tracee (e.g. glucose) of interest in that pool. If the pool size is large relative to flux of the tracee, a priming dose can be used to label the sampling pool with the tracee, to reduce the infusion time needed to achieve tracer: tracee steady state at detectable concentrations.

A fundamental principle of using stable isotopes is that the label used in a given tracer does not re-appear in the sampling pool after it has been removed with the tracee as re-cycling would result in the rate of appearance of the tracee being underestimated (29). The total rate of blood glucose appearance is typically determined using hydrogen (^{2}H [deuterium] or ^{3}H [tritium]) as opposed to carbon isotopes. In **Chapter 3**, [6, 6-$^{2}H_2$]-glucose was chosen as an infusate, where two hydrogen atoms attached to carbons at position 6 are deuterium. This is because only carbon-labeled glucose can be recycled during gluconeogenesis (GNG) of pyruvate and lactate from the Cori cycle (386). Hydrogen isotopes can be labelled on the 2nd, 3rd or 6th carbon, with this label lost to water in the various steps of

glucose metabolism. These processes can occur in the liver, which also has the enymes needed to reverse each stage of glucose phosphorylation. Thus, whilst GNG may not be a consideration with a hydrogen *versus* a cabon label, it is still possible to underestimate hepatic glucose production (HGP) with recycling of the hydrogen label in the liver. If glucose is labelled on the 2nd carbon, the hydrogen label is lost in the conversion of glucose-6-phosphate to fructose-6-phosphate and labelling on the 3rd carbon is lost with cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. However, when glucose is labelled on the 6th carbon, the label can only be lost after entry into the TCA cycle, which is non-reversable (387). Blood glucose appearance rates from [6,6-^{2}H]-glucose therefore represents the appearance of glucose from hepatic glycogen or GNG and not glucose cycle activity, as the label on the 6th carbon cannot be lost in reversible steps of glucose metabolism. Using [6,6-$^{2}H_2$]-glucose as a tracer helps to overcome issues with futile cycling as this tracer can be distinguished from the [6-$^{2}H_1$]-glucose product of recycling in analysis (388).

Combining a [6,6-$^{2}H_2$]-glucose infusion with the oral ingestion of a second tracer also provides data on the appearance of glucose from a meal. First, the infused tracer can be used to estimate the total rate of blood glucose appearance and the appearance of the ingested glucose can be estimated by multiplying the rate of appearance of the ingested tracer in the plasma by the tracer: tracee ratio of the meal. HGP is calculated as the total glucose appearance rate minus the rate of appearance of ingested glucose. Finally, plasma glucose disposal rates can be estimated by substracting the change in blood glucose concentrations from the total rate of glucose appearance. Universally labelled [U^{13}-C]-glucose was the chosen oral tracer in **Chapter 3**, because there is no net loss of carbon during glycolysis, so this *versus* a hydrogen label avoids issues with label recycling in the liver. Using [U-^{13}C]-glucose instead of an isotope with fewer carbon labels also helps to avoid issues with recycling from GNG as for universally labelled glucose to reappear in the blood, two triply labeled 3-carbon precursors would need to have combined to form [U-^{13}C]-glucose (389). Choosing an appropriate infusion rate is also important because the tracer must be detectable but not alter

the endogenous metabolism of the tracee of interest and the infusion rate also dictates the quantity and cost of the tracer required. In **Chapter 3**, the prime and infusion rates were based on prior research with a similar participant cohort and protocol (390). Previous work was also used to inform the decision to modify the infusion rate during exercise (391) and the OGTT (390) to account for expected changes in HGP and to reduce alterations in the tracer-to-tracee ratio in order to obtain more accurate measures of glucose flux (29).

Preparation of tracers

For the OGTT, 73 g of glucose was first weighed as 81 g of dextrose monohydrate corrected for water content (Myprotein, Northwich, UK). Then, 2 g of [U^{13}-C]-glucose (99 %; Cambridge Isotope Laboratories, MA, USA) was weighed to the nearest 0.01 g with electronic weighing scales (AX124/E Adventurer Analytical Balance, Ohaus, Parsippany, USA) and added with 300 mL of plain water. The infusate and prime were freshly made for each trial. A spreadsheet was prepared with desired tracer weights and saline volumes which were calculated from the desired infusion rate, the MW of [6,6-$^2H_2$]-glucose, the MW of sodium chloride, the expected length of the infusion, an estimation for the volume of distribution of glucose in the blood as 4 % of the participants body mass and an estimated fasting plasma glucose concentration of 4.5 mmol·L^{-1}. Tracer preperation was completed under a sterlie fume hood and work surfaces, material packaging and equipment were sprayed with 70 % ethanol. The desrired mass of [6,6-$^2H_2$]-glucose was weighed seperately for infusates and primes to the nearest 0.01 g and the actual mass was recorded. Then, a needle was attached to a 60 mL sterile syringe and used to add the desired volume of saline for the infusate and prime and the actual volume was recorded. A second syringe was used to aspirate the solution for the infusate and prime and was attached to a 3-way stopcock, a 0.2 micron syringe filter and an intravenous infusion extension line (for the prime, the syringe was only attached to the filter). The spreadsheet was updated with the actual weights recorded so that the required pump rate could be calculated. During trials the infusion rate was administered via an automatic pump

(Alaris™ PK Syringe Pump, BD, Wokingham, UK) and the infusate syringe was weighed after each trial to ensure the infusion rate prescribed was accurate.

Sample preparation

Plasma [U^{13}C]-glucose and [$^{2}H_2$]-glucose enrichments were determined via gas chromatography-mass spectrometry (GC-MS). First, the glucose was extracted from samples using methanol-chloroform and hydrochloric acid, dried under nitrogen gas and derivatised to ensure samples were sufficiently volatile and thermally stable for GC-MS analysis (392). Specifically, 150 µL of plasma was added to 3 mL of a methanol: chloroform mixture (2.3:1 ratio), vortexed for 10 min using a plate shaker (MidiMix, Denley) and left on ice for 10 min. This was then centrifuged at 3000 *g* and 4 ºC for 15 min (IEC Centra-CL3R, Thermo Electron Corporation, Massachusetts, US). The top layer containing glycerol, glucose and NEFA was removed using a pipette and transferred into a new tube (borosilicate glass and threaded end with phenolic caps; Thermo Fisher Scientific, Massachusetts, US). Thereafter, 2 mL of chloroform and 1 mL of hydrochloric acid was added, before 10 min of vortexing and centrifuging at 3000 *g* at 4ºC for 10 min. The top layer with glycerol and glucose was transferred into a new tube and the supernatant was evaporated under nitrogen at 70 ºC (Multivap 118 Nitrogen Evaporator; Organomation, Massachusetts, US and QBT4 dry block heating system; Grant Instruments, Cambridge, UK). The pellet produced was resuspended in 200 µL of a 1:3 hexafluorobenzene: ethyl acetate solution and incubated at 70 ºC for 10 min. The solution was centrifuged at 3000 *g* and 25 ºC for 5 min and evaporated under nitrogen. The sediment was resuspended in 100 µL of ethyl acetate, vortexed for 10 min, left at room temperature for 15 min and transferred to a vial for GC (glass inserts; Macherey-Nagel, Düren, Germany).

Gas chromatography mass spectrometry (GC-MS)

The [6,6-$^{2}H_2$]- and [U-^{13}C]-glucose enrichments were determined using Agilent Technologies gas chromatography and mass selective detectors (GC; Agilent Technologies 6890N, UK; mass selective detector; Agilent Technologies 5973). The sample was injected into the GC inlet where it was vaporised so that the

resultant gases could be carried down the chromatographic column by a carrier gas and the different substances in the sample could be separated according to their interactions with the coating of the column and carrier gas. The separated substances emerged from the column into the mass spectrometer, where they were ionized and separated according to their mass-to-charge (*m/z*) ratios. Data analysis was completed on commercially available software (MSD ChemStation analysis, Cheshire, UK). The glucose derivatives were acquired by using selected ion monitoring at *m*/*z* 519, 521 and 525 for [^{12}-C]-, [6,-6$^{2}H_2$]-, and [U^{13}-C]-glucose, respectively and the area under each peak was calculated. Corrections were made for the order in which the samples were loaded onto the GC and the plasma enrichments of the isotopes were calculated via standard curves for [^{13}-C]- and [$^{2}H_2$]-glucose and expressed relative to enrichments for [^{12}-C]-glucose. A baseline sample from every trial was used to account for background isotopic enrichments.

Data modelling and calculations

To reduce any impact of analytical variability on calculations of plasma glucose flux, plasma glucose concentrations and enrichments were curve fitted using a 3rd Order Polynomial (393). Plasma glucose flux was assessed with Radziuk's two-compartment non-steady state model (394, 395). First, the total plasma glucose appearance (Ra_{TOTAL}) and disposal rates (Rd) were calculated as:

$$1)\quad Ra_{TOTAL}(t) = \frac{F}{E_1(t)} - \frac{V_1 \cdot G(t)}{E_1(t)} \cdot \dot{E}_1(t) + k_{12}\left(\frac{q_2^{iv}(t)}{E_1(t)} - Q_2(t)\right)$$

$$2)\quad Rd\,(t) = Ra_{TOTAL} - V_1 \cdot \dot{G}(t) - k_{21} \cdot V_1 \cdot G(t) + k_{12} \cdot Q_2(t)$$

Where F is the [6,6-$^{2}H_2$] infusion, V_1 is the glucose volume of distribution [4 % of the participant's body mass as this results in less variable estimations of glucose flux for an OGTT compared to if larger values are used (29)], $E_1(t)$ is the [$^{2}H_2$]-glucose enrichment (mole percent excess) at time *t*, $\dot{E}_1(t)$ is the change in E over time (derivate of E), G*(t)* is the plasma glucose concentration at time t, $\dot{G}(t)$ is the change in G over time (derivate of G), k_{12} and k_{21} are fixed constants between the peripheral and sampling pools (0.05 and 0.07 respectively) and q_2^{iv} and Q_2

are the amounts of tracer and tracee in the peripheral compartment respectively, evaluated by integrating the two-compartment model.

The [$U^{13}C$]-glucose enrichment of the OGTT and Ra_{TOTAL} were then used to assess the rate of appearance of glucose from the OGTT (Ra_{OGTT}) in plasma. In the subsequent equations, r_1 represents the ratio of the infusion [$^2H_2$] and oral [$U^{13}C$]-glucose tracer enrichments in plasma, $\dot{r}(t)$ is the change in r over time (derivate of r), g is the [$U^{13}C$]-glucose in plasma, q_o^{iv} is the amount of [$U^{13}C$]-glucose in the peripheral pool (evaluated by integrating the two-compartment model) and E_{OGTT} is the OGTT [$U^{13}C$]-glucose enrichment.

$$3)\quad ra^O(t) = Ra_{TOTAL}(t)\frac{F}{r_1(t)} - \frac{V_1 \cdot g(t)}{r_1(t)} \cdot \dot{r}_1(t) + k_{12}\left[\frac{q_2^{iv}(t)}{r_1(t)} - q_2^0(t)\right]$$

$$4)\quad Ra_{OGTT}(t) = ra^O(t)\left[\frac{1}{E_{OGTT}}\right]$$

In the 1950's and 60's Steele *et al* developed dual tracer methods for studying glucose flux in a single-pool model (396-398). Since then, others have used the principle of the method to study glucose flux but have addressed some limitations associated with a single pool model. The problem with the single-pool model, where the tracee and the tracer are uniformly distributed in a steady state, is that this does not represent many physiological scenarios. For example, during an OGTT there is a large influx of glucose into the blood and the glucose crosses, and can be extracted, by many tissues prior to this (399, 400). Thus, at least two pools are present for glucose in this condition; a rapidly equilibrating inaccessible pool and the sampling pool (plasma). A failure to account for this second pool increases errors in the estimations of glucose flux and especially HGP (399, 400). A potential solution to this problem is the use of a pool fraction, which assesses the sampling pool as a fraction of the volume of distribution of glucose in the body, but the most appropriate value for a pool fraction can also vary under non-steady state conditions (401). Alternatively, using a model with an accessible and non-accessible pool reduces errors with estimating plasma glucose flux, especially when combined with an infusion rate that is adjusted for anticipated changes in

HGP (401). A triple-tracer approach has also been used previously, where in addition to an orally ingested tracer, two intravenous tracers (e.g. [6-^{3}H]-glucose and [6, 6-$^{2}H_2$]-glucose) are infused in patterns that minimise the change in their ratio to the ingested tracer and glucose via HGP, respectively. Thus, calculations of HGP become independent of modelling (400). However, there are large costs with the purchase and analysis of a third tracer, and the dual tracer method when implemented carefully can provides accurate estimations of plasma glucose flux.

MUSCLE SAMPLING AND ANALYSIS

Throughout this thesis, skeletal muscle was sampled and primarily analysed for the content and/or activity of proteins involved in glucose and/or lipid metabolism. Samples were collected (~ 100 mg of wet weight) from the *vastus lateralis* using a Bergstrom biopsy needle technique, which was adapted for suction (402) and from separate skin incision sites, with these > 2 cm proximal to any prior incisions on the same leg. Upon arrival at the laboratory participants rested for 15-20 min in the supine position. The skin surrounding the sampling site was shaved before being cleaned and sterilised with iodine (Videne, EcoLab, UK). A local anaesthetic (2.5 mL of 1 % lidocaine, Hameln Pharmaceuticals Ltd., UK) was injected under the skin using a 27 G needle and a second 2.5 mL dose of lidocaine was injected onto the outside of the muscle facia (2-5 cm beneath the skin surface) using a 21 G needle. A 3 mm incision at the anterior aspect of the thigh was made with a blade (Swann Morton, size 11), iodine was applied and pressure placed on the incision. Samples were taken using a 5-mm Bergstrom biopsy needle (402) which was vertically inserted into the incision to a depth of at least 1 cm beyond the fascia. A vacuum was created using a 100 mL syringe attached to the needle to allow for the muscle to be snipped and this was repeated 2-3 times to obtain a sample. After removal of the needle, the incision was sealed with a proline stitch and steri-strips, then covered with a water-proof adhesive dressing. Samples were extracted from the needle and cleaned in saline with dissection of obvious blood or connective tissue. Cleaned samples were placed into ventilated Eppendorf tubes in liquid nitrogen, before storage at -80°C.

Powdering

Upon removal from storage and whilst still immersed in liquid nitrogen, each sample was dissected to remove a fragment of muscle tissue (20-30 mg), which was freeze-dried for 12 h at -40 °C and -10^{-1} mmHg (Edwards Modulyo; Thermo Fisher Scientific, Massachusetts, US). Thereafter, any remaining visible blood or connective tissue was removed with a small blade (Swann Morton, stainless steel, size 11) before samples were powdered with an agate pestle and mortar. Powder was divided into separate Eppendorf tubes and weighed to the nearest 0.01 g (2 mg dry weight per tube) using an analytical balance scale (Sartorius AG, Göttingen, Germany), before powdered samples were again stored at -80°C.

Assessment of protein content

Powdered samples were added, at 100 $\mu L \cdot mg^{-1}$, to ice cold lysis buffer as detailed in the final sub-section of **Chapter 2**, with phosphatase (Thermo Fisher Scientific, Massachusetts, US) and protease inhibitors (Merck Millipore, Massachusetts, US). Samples were then homogenised with 30-40 passes of a hand-held dounce homogeniser (VWR International Ltd, Leicester, UK), incubated for 60 min at 4°C with rotation (Gallenkamp Vacuum Oven, Fistreem International Ltd, Leicester, UK) and centrifuged for 10 min at 4°C and at 20,000 *g* (Eppendorf™ 5424 Microcentrifuge, Hamburg, Germany) to remove insoluble material. The protein content of the resultant supernatant was measured using a bicinchoninic acid assay. Samples were diluted at a ratio of 1:10 using 0.1 M sodium hydroxide (NaOH) and a standard curve was freshly prepared with dilutions of a 1 $mg \cdot mL^{-1}$ bovine serum albumin (BSA) stock. Then, 10 µL of the standards and samples were pipetted in duplicate onto a 96-well plate. To each well, 196 µL protein assay reagent A (Thermo Fisher Scientific) and 4 µL copper(II) sulfate was added, before a 30 min incubation at 37°C. This assay relies on two reactions; first, the peptide bonds in protein reduce Cu2+ ions from the copper(II) sulfate to Cu+. The amount of Cu2+ reduced is proportional to the amount of protein in the original sample. Next, two molecules of BCA chelate with each Cu+ ion, which forms a purple-coloured complex. The intensity of the colour produced in this reaction is determined by reading the absorbance at 562 nm (SPECTROstar plate reader,

BMG Labtech, Aylesbury, UK). The protein content of each sample was then calculated by comparing the obtained absorbance values to the standard curve.

Western blots

Western blotting was first developed in 1979 and provides semi-quantitative data about the content and/or activity status of proteins in a sample (403). First, the different proteins in a sample were separated via gel electrophoresis. Linear tris-glycine sodium dodecyl sulphate (SDS)-polyacrylamide gels were casted in CBS (VWR) gel cassettes to obtain 10 % polyacrylamide resolving gel overlaid with a 6% polyacrylamide stacking gel. Then, an equal amount of protein (~ 40 µg) was loaded for each sample in a 50 µL volume into separate wells, with 8 µL of MW markers (Thermo Fisher Scientific) in one well. Proteins were separated according to their MW by SDS-polyacrylamide gel electrophoresis using an electrical current applied at 200 V for ~ 1 h, whilst the gel was submerged in electrophoresis buffer. Proteins were then electro-transferred using a semi-dry transfer onto a nitrocellulose membrane (Gelman Sciences, Portsmouth, UK). This uses the electrophoretic mobility of the proteins in order to transfer them from the gel onto the membrane which is sandwiched between electrodes with the aid of a conducting solution (SDS-Transfer Buffer). The nitrocellulose membrane and filter papers (Bio-Rad Laboratories; Hercules, California, USA) were cut to an appropriate size and soaked in SDS-transfer buffer, before being placed on a transfer unit (Bio-Rad Laboratories; Hercules, California, USA). An electric current was created at 75 mA to facilitate the movement of the proteins from the gel onto the membrane. Membranes were washed to remove any transfer buffer before staining in a 0.1% Ponceau S solution in 3 % Trichloroacetic acid (TCA) and imaging using a chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, US) to assess transfer efficiency. Then, membranes were washed in Tris-buffered saline (TBS) and cut according to the MW of the protein of interest and incubated for 30 min in a blocking solution (5 % non-fat dry milk in TBS-T; Marvel, Premier International Foods Ltd, UK) to reduce nonspecific binding of the detection antibody. This was followed by washing (4 x 5 min) to remove the blocking solution. Thereafter, membranes were incubated overnight

for ~ 12 h at 4°C with primary antibodies diluted ~ 1:1000 in TBS-T with 1 % BSA, against the protein of interest. All of the primary antibodies used and the suppliers are detailed in the relevant chapters of this thesis. Thereafter, membranes were washed with TBS-T (5 x 5 min) and incubated in anti-species IgG horseradish peroxidase-conjugated secondary antibodies (1:4000 dilution) in the blocking solution. The secondary antibody used depends on the animal in which the primary antibody was raised. For example, for a mouse monoclonal antibody, an anti-mouse IgG secondary antibody from a non-mouse host is combined with horseradish peroxidase, which reacts with the chemiluminescence reagent.

Membranes were then visualised on the chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, US). Multiple images of the membranes were made with a CCD cooled camera, which measures the quantity of light produced from the chemiluminescent reaction. Images were taken with various exposure times to allow capturing images with optimal intensity of the protein bands. The volume of pixels in each band were quantified using appropriate software (as detailed in the relevant chapters of this thesis). Images were inverted to produce an image with black bands on a white background and the intensity of the picture was adjusted to allow the bands of interest to be more identified easily. For each band, a background correction was completed at ~ 10 % of the intensity of each band, with manual adjustments made to ensure that the entire width and peak of each band was accounted for in the analysis. Due to the semi-quantitative nature of the method all samples from a participant were included on the same gel and quantification was done using the same software and by the same researcher. After imaging, membranes were sometimes incubated for 30 min at 50°C in a stripping solution (62.5 mM Tris pH 6.7, 2 %SDS, 100 mM 2-mercaptoethanol) to allow for re-blotting of the same membrane with different primary antibodies.

ENERGY BALANCE

Assessing energy intake

In **Chapter 5**, energy intake was assessed over 24 h including within-laboratory and free-living periods. Participants were given *ab libitum* researcher-weighed

meals for lunch within-laboratory and dinner (free-living). Meals were designed to be homogenous to reduce the likelihood that a participant would overconsume or preferentially consume one macronutrient (404). The macronutrient composition of the meals was selected to reflect the high-carbohydrate diet typical to developed countries (186). For within-laboratory meals, fresh portions were continually provided to ensure that the participant finishing one portion was not responsible for meal termination, and they were asked to eat until comfortably full. For meals outside of the laboratory, participants were instructed to eat until comfortably full, not to eat and/or drink anything not provided in the food package (except water) and to bring remaining food back to the laboratory. For all meals, the carbohydrate, fat and protein content (g) was taken from the manufacturer's label. Intake of a macronutrient was calculated as the g provided (cooked weight) minus the g remaining after the meal. The energy consumed was estimated with carbohydrates, fats and proteins giving 3.74, 9.42 and 4.09 $kcal \cdot g^{-1}$, respectively. These values are equivalent to values that were used for the energy expended with the utilisation of each substrate, so that calculations of energy/macronutrient balances would be the same if expressed as g or kcal.

In **Chapter 6**, participants were asked to keep a weighed diet record of their food and fluid intake over a typical 4-day period at baseline and during the final week of the intervention. The importance of maintaining their habitual diet during the baseline monitoring period was outlined to participants and they were provided the following instructions verbally and in writing; *"Your lifestyle choices during this free-living monitoring period are central to this study. We are interested in any natural changes in your diet and/or physical activity habits, which you may or may not make in response to the intervention. This monitoring period has been carefully scheduled to avoid any pre-planned changes in these habits, such as a holiday or diet/exercise plan. You should inform us immediately if unforeseen factors external to the study may influence your lifestyle."* As variation exists between reported weekday and weekend energy intakes, a minimum of one weekend day was included in the monitoring periods (405). Each participant received weighing scales (ARC Electronic Kitchen Scale; Salter; Kent, UK) to

help reduce errors in estimations of food weights and they were asked to provide packaging or photos of meals if weighing was impractical. Records were analysed on Nutritics software for macronutrient intakes (Nutritics Ltd., Dublin, Ireland). The composition of each food item was obtained from the manufacturer's labels and if this was not possible, the foods were analysed using the software database, or comparable brands were used to provide necessary information and this was kept constant across all records for a given participant. An average daily intake of each macronutrient was calculated for the monitoring period and was then used to calculate energy intake using aforementioned values for carbohydrate, fats and proteins and with alcohol providing 7.10 $kcal \cdot g^{-1}$ (406).

Accurately assessing energy intake in humans is one of the most difficult aspects of energy balance research, because most methods depend on self-reporting, which introduces observational, reporting and recall biases (407). In a laboratory when researchers prepare and weigh food, it is possible to assess energy and macronutrient intakes to the nearest ingested gram without any self-reporting. However, even this method can be cofounded by observational biases, as energy intakes can be 5-10 % lower if a participant is aware of being observed, in line with social desirability to not over-consume (408). Providing researcher-weighed meals is also less feasible in a free-living environment (407). One retrospective method is the food frequency questionnaire, which asks questions about prior meals (the frequency and portion size) over a given time period and often from a pre-determined list of foods, although the list of foods and timescale used for the assessment varies across studies (407). As this method is inexpensive and has a low participant burden, it is typically used in epidemiological research, but it is associated with issues such as a limited list of foods. A more personal approach is where a participant interacts with an interviewer with the aim of capturing their food and fluid intake and cooking methods from a previous day (24-h recall) or a longer period. This typically provides more information than a food frequency questionnaire but can still be influenced by recall bias and relies on estimated portion sizes. To help avoid this potential source of error, participants are often asked to complete weighed records of their food and fluid intake. The optimal

cited timescale is 7 days (to help account for day-to-day variability in energy intakes) but the relatively high burden associated with recording often leads to under-reporting or under-eating, so shorter records have also been used in an attempt to improve the accuracy of diet records (405). The weighed diet record method overcomes issues with recall bias, but dietary changes can occur (407). Participants may consume pre-prepared food due to the ease of recording this (compared to weighing ingredients) and their responses and/or diet may change in line with social desirability (407).

Validating methods of assessing energy intake is also difficult because every measure is subjected to some bias. However, one study used covert observation as a control, compared to weighed diet records, 24-h dietary recall and 12-day food frequency questionnaires (407). The study was separated into phases when the participants did not monitor energy intake and when they completed 3-day weighed diet records and dietary recalls. However, researchers monitored energy intakes throughout the whole study and the participants were not informed of this. Based on a principle that energy cannot be created or destroyed, body mass responds to changes in energy intake *versus* energy expenditure (18). To confirm the validity of the energy intake data from covert observation, body mass and energy expenditure via doubly-labelled water (as discussed subsequently in this review) were assessed. There was no difference between the measured energy expenditure and covertly-assessed energy intake and body mass also remained unchanged, suggesting that the observation method could be used as a valid control during that study. A 5.3 % reduction for energy intake occurred in the overt *versus* the covert observation phases, supporting the idea that energy intakes are lower when a participant is aware of being observed (408). Relative to covert observation, the energy-intakes were decreased by ~ 5 % for the weighed diet records ~ 10 % for 24-h diet recall and ~ 15 % for food frequency questionnaire and 7-day diet recall. The magnitude of under-reporting for energy intake is also often greater in humans with obesity and with increasing socioeconomic status (409). Biomarkers such as blood lipid or vitamin concentrations have also been used to estimate the dietary consumption of some foods to avoid issues with self-

reporting, but this is not currently possible for many foods (410). Finally, as eluded to previously, when energy expenditure and body mass are measured over time, inferences can be made about energy intake via a rearrangement of the energy balance equation, but this does not allow macronutrient intakes to be assessed (409). To conclude, although all methods available for assessing macronutrient intakes are subjected to some biases, when assessed in a laboratory, providing researcher-weighed meals without observing the participant provides the most accurate data about energy and macronutrient intakes. In a free-living setting, a weighed diet record is considered the most valid approach to measure energy intakes and will be most accurate if the participants are aware of the importance of accurately recording their food and fluid intakes (409).

Assessing energy expenditure

In this thesis, total daily energy expenditure was measured as the summation of resting metabolic rate (RMR), diet induced thermogenesis (DIT) and physical activity energy expenditure (PAEE). RMR was assessed via indirect calorimetry from a minumum of 3 x 5 min expired air samples collected into Douglas bags. The participant rested in a semi-supine position for 15 min before the first sample in accordance with the guidelines for measuring RMR (411). DIT was assumed to be 10 % of recorded or measured energy intake (338). For within-laboratory periods, PAEE was calculated as energy expenditure from indirect calorimetry minus RMR. During free-living periods, PAEE was assessed with an Actiheart^{TM} monitor. The unit was fitted through adhesive ECG chest electrodes or a strap and participants wore the monitor from 23:30 on the day prior to a measurement period. If the monitor was not worn (e.g. when swimming) participants recorded any physical activity performed. The monitor was initialised to long-term recording with 1-min epochs and PAEE was calculated via branched-equation modelling. Stature, body mass, age, sex and sleeping HR were entered in the software for each participant. Mean sleeping HR was calculated by excluding the lowest and highest recorded sleeping HR for a monitoring period, averaging the remaining days and excluding days when the measured value was ± 10 % of this average. For each participant, measured PAEE and HR values from rest and exercise were

entered into the software to derive an individually calibrated model, which improves PAEE estimations (412, 413). Data were only considered to be of useable quality and included in any subsequent analysis if > 90 % of the activity trace was 'not lost' (i.e. non-wear time) with < 30 % of the HR trace extrapolated by the software, whereby a 75 % weighting of PAEE towards accelerometry. In **Chapter 6**, participants wore the Actiheart^{TM} for 7-days as this period achieves an 80 % reliability in activity counts for the monitor.

Assessing energy expenditure involves measuring reactants and/or products of the metabolism of glucose, lipid and protein molecules (88).

1) 1 g Glucose + 0.746 L O_2 → 0.746 L CO_2 + 0.6 g H_2O + Energy
2) 1 g Lipid + 2.029 L O_2 → 1.430 L CO_2 + 1.09 g H_2O + Energy
3) 1 g Protein + 0.966 L O_2 → 0.782 L CO_2 + 0.45 g H_2O + Energy

However, these reactions are not 100 % efficient so some of the energy liberated is lost as heat, instead of being transferred to ATP. This underpins the principle of direct calorimetry, which is that the amount of heat lost by an organism at a given time is proportional to the amount of energy being expended (414). Direct calorimeters facilitate heat exchange within the system but not between the system and the environment with water and air used as the mediums to allow heat transfer, so that changes in their temperature can then be equated to an energy parameter (414). Specialist and expensive equipment is however required and although large chambers have been used to assess energy expenditure, they are not most representative of free-living settings and can exert confounding influences on activity behaviours (414). An alternative method is indirect calorimetry, when oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) are assessed from expired gas samples and sometimes combined with measures of urinary nitrogen excretion. The principle of this method is that the fate of ingested substrates is their oxidation to resynthesize ATP and to liberate energy (88, 368). The oxidation of a given mass of glucose, fat or protein will utilise a known quantity of O_2 and produce a known quantity of CO_2, and at the whole-

body level $\dot{V}O_2$ and $\dot{V}CO_2$ reflects O_2 consumed and CO_2 produced from metabolism of all substrates at a given time. Analysis can then be completed to isolate a contribution of each substrate to metabolism. It is first possible to isolate a contribution of protein by measuring nitrogen excreted in urine as discussed previously and shown in **equation 4**. This can be substituted into **equation 3** to derive CO_2 produced and O_2 consumed per g of nitrogen excreted (**equation 5**).

4) Protein utilisation (g) = 6.25 x urinary nitrogen excretion (g)

5) 1 g Nitrogen [N] (g) + 6.038 L O_2 → 4.888 L CO_2 + 2.81 g H_2O + Energy

A 1:1 ratio of CO_2 produced to O_2 consumed is apparent with the utilisation of glucose (88). Therefore, if the contribution of protein to metabolism is known or if protein utilisation is assumed to be negligible any discrepancy between $\dot{V}O_2$ and $\dot{V}CO_2$ must be explained by the utilisation of lipids and this has a ratio of 1: 0.695 for O_2 consumed: CO_2 produced. As such, the amount of lipid being utilised can be determined from $\dot{V}O_2$ and $\dot{V}CO_2$ measures, before this value is then used to calculate the contribution of carbohydrate to energy metabolism. This is outlined in the subsequent equations, where **equation 7** is subtracted from **equation 6** for **equation 8**, which can be rearranged for **equation 9**, before this is substituted into **equation 6** to give **equation 10**, before rearranging to give **equation 11**.

6) $\dot{V}O_2$= (0.746 x CHO [g]) + (2.029 x lipid [g]) + (6.038 x N [g])

7) $\dot{V}CO_2$ =(0.746 x CHO [g]) + (1.430 x lipid [g]) + (4.888 x N [g])

8) $\dot{V}O_2 - \dot{V}CO_2$= (0.599 x lipid [g]) + (1.150 x N [g])

9) Lipid utilisation (g) = 1.67 $(\dot{V}O_2 - \dot{V}CO_2)$ - (1.92 x N [g])

10) $\dot{V}O_2$= (0.746 x CHO [g]) + $(3.388 \times \dot{V}O_2)$ - $(3.388 \times \dot{V}CO_2)$ - (2.144 x N [g])

11) CHO utilisation (g) = $(4.542 \times \dot{V}CO_2)$ - $(3.201 \times \dot{V}O_2)$ + (2.874 x N [g])

Once the amount of each substrate (g) that has been oxidised over a given time period is determined, total energy expenditure can be calculated by multiplying the amount of each substrate oxidised (g) by their caloric equivalents. Indirect calorimetry can therefore provide insights into energy expenditure and also the

relative contribution of each substrate. However, the method relies on several assumptions, including that $\dot{V}O_2$ and $\dot{V}CO_2$ is only attributable to these oxidation reactions and as such any non-metabolic processes which consume or produce O_2 and/or CO_2 or any inter-conversion of substrates will induce some error (67). The most important of these assumptions are highlighted prior to **Chapter 5** of this thesis in relation to the data presented in that experimental chapter.

Different methods use the principles of indirect calorimetry to assess substrate and energy metabolism. Respiratory chambers can accurately measure $\dot{V}O_2$ and $\dot{V}CO_2$ over several days but have cost and space constraints that are similar to limitations for direct calorimetry (415). The Douglas bag method can also be used to provide the same data without the impracticalities of cost and space but this is still limited to laboratory settings (415, 416). Automated portable systems assess expired and inspired O_2 and CO_2 concentrations and equate this to a volume using an inline flow sensor (414). This method permits a greater flexibility in the range of activities that can be assessed, but is still quite constrained to settings that do not represent free-living physiology. Therefore, whilst these methods can give accurate measurements of $\dot{V}O_2$ and $\dot{V}CO_2$, they are often restricted to the controlled environments where energy expenditure can be assessed (415). This is not a problem when assessing energy expenditure during rest or diet-induced thermogenesis, but any measures of physical activity energy expenditure from these methods should be interpreted in the context of a laboratory environment.

The criterion method for assessing energy expenditure in a free living setting is doubly-labelled water, which uses isotope tracers to assess $\dot{V}CO_2$ (417). When combined with a set value for the respiratory exchange ratio of typically 0.85, this method can estimate total energy expenditure over several days. Following the ingestion of water composed of hydrogen (H) and O_2 isotopes, several hours are allowed for the isotopes to reach equilibrium in the body water pool before a baseline (saliva, urine or blood) sample is collected. Over time, H and some O_2 isotopes are lost as water, but some of the O_2 atoms are incorporated into CO_2 molecules during metabolism. As such, the O_2 isotope is depleted at a quicker

rate than the H isotope and the difference is due to CO_2 production. Therefore, measuring the depletion of the isotopes in subsequent samples of the body water pool allows free-living energy expenditure to be estimated (417). One limitation of the method is that it does not account for losses of the isotopes into molecules other than water or CO_2 and it does not account for changes in enrichment from dietary changes, although these are likely to have a negligible effect under normal physiological circumstances. However, the isotopes are also relatively costly and without sampling at frequent intervals the data resolution is low, meaning that any inferences about patterns or timing of physical activity are difficult to make (417). Any change to the RER away from 0.85 with feeding and/or exercise can also introduce errors, unless RER is frequently measured and accounted for.

Recent technological advancements have facilitated a development of wearable sensors that are now more commonly used to assess free-living physical activity energy expenditure. By measuring accelerative forces, accelerometers can give information about the duration, frequency and intensity of movements. However, in isolation, accelerometers typically perform poorly against doubly-labelled water for assessing energy expenditure (418). An alternative method to estimate energy expenditure is by measuring HR, which is grounded on the linear relationship between HR and $\dot{V}O_2$ (419). However, this method is also subject to limitations, including inter-individual variation in the HR-$\dot{V}O_2$ relationship, due to differences between people in, for example, age, fitness and the relative efficiency of the movements being recorded (412). When an individual calibration of the HR-$\dot{V}O_2$ relationship is performed, the agreement with doubly-labelled water derived estimates of energy expenditure is improved (420). Despite this, HR can still be influenced by other factors such as environmental temperature, hydration status and stress which can result in errors in the energy expenditure estimations in a free-living environment. Monitors have also been developed that incorporate HR and accelerometry by using branched-equation modelling for energy expenditure assessments (420). Energy expenditure estimations can be made by classifying each min of activity into a category (i.e. low movement and HR, low movement but high HR, high movement but low HR or high movement

and HR) and weighting the estimate of energy expenditure towards either the HR monitor or the accelerometer, accordingly (420). The Actiheart™ utilises a chest-worn HR sensor and an accelerometer to measure energy expenditure (421). To initially validate this device, energy expenditure estimations for walking and running were compared to indirect calorimetry. Combined HR-accelerometry showed a better agreement with indirect calorimetry than either sensor used in isolation and the standard error of the estimate was reduced by individualised calibrations (412, 421). Estimations of energy expenditure from an Actiheart™ are similar to those derived from doubly-labelled water (413) and with the monitor information can also be obtained about the timing or intensity of physical activity.

STATISTICAL ANALYSIS

An account of the statistical approach for each study is detailed in the relevant chapter, but common methods are outlined here. Analyses was completed on commercially available packages (IBM SPSS statistics V22 for windows, New York, USA; Graph Pad Prism 7, La Jolla, CA, USA; and Microsoft Excel 2013). Sample size estimations were made *a priori*, according to the outcomes of a given study and were calculated to provide at least an 80 % probability of correctly rejecting a null hypothesis. Statistical significance was accepted at $p \leq 0.05$.

The total or incremental area underneath the concentration-time curve for plasma metabolites and hormones were calculated using the trapezoid rule (422). These values were divided by specific time periods for summary values. Data requiring a single comparison of two means (paired differences for repeated measures) were normality tested using Shapiro-Wilk tests. A *t*-test with a 2-sided significance level was then applied for normally distributed data and non-normal data was assessed with a Wilcoxon test or was log-transformed before analysis. If multiple comparisons were necessary, two-way repeated measures or mixed-model ANOVAs were used to assess any differences between trials or groups. This was regardless of normality, as the type 1 error rate of ANOVA remains at $p \leq 0.05$, even when the data are non-normally distributed within a physiological range (423). Degrees of freedom for *F* values were Greenhouse-Geisser corrected for

epsilon <0.75, with Huynh-Feldt corrections made for less severe asphericity. If interaction effects (e.g. time x trial) were identified, *t*-tests were used to locate variance between trials or groups, with Holm-Bonferroni step-wise adjustments made to control for type I errors (424). Unless otherwise stated, all data in text, figures and tables are means ± 95 % confidence intervals.

Confidence intervals were normalised to remove between-subject variance from an equation using the mean square error value from the corresponding statistical general linear model if this was appropriate (425). These confidence intervals are more reflective of differences between group or trial means than the variance of individual participants around the mean. If data sets were incomplete, results were sometimes extrapolated from measured values with the last observation carried forward approach, but sensitivity analysis was completed with this data included or excluded. For clarity, the *n* is presented in all figure and table legends.

BUFFERS AND STOCK SOLUTIONS FOR WESTERN BLOTTING

The following stock solutions were made in batch for western blot analysis and were stored for up a maximum of two months and at room temperature. Unless stated chemicals were purchased from Thermo Fisher Scientific (Massachusetts, US) or Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK).

Lysis (RIPA) buffer: 1x concentrated stock

	From Stock	**1 x (50 ml)**
50 mM Tris (pH 7.4)	1 M Tris pH 7.4	2.5 mL
150 mM NaCl	2.5 M NaCl	3.0 mL
0.5 % Sodium deoxycholate		250 mg
0.1 % SDS	10 % SDS	500 µL
0.1 % NP-40	100 % NP-40	

SDS-sample buffer: 3x concentrated stock, final pH 6.8

187.5 mM Tris, 6 % SDS, 30 % glycerol

Stacking Gel	*Resolving Gel*				
	7 %	**8%**	**10 %**	**12 %**	**6%**
Acrylamide stock solution (mL)	17.5	20	25	30	3.4
Resolving gel buffer (mL)	25	25	25	25	-
Stacking Buffer (mL)		-	-	-	5.0
Double-distilled water (mL)	32.5	30	25	20	7.5
Ammonium persulphate (µL)	500	500	500	500	100
TEMED (mL)	40	40	40	40	20

Resolving gel buffer: 1.5 M Tris hydrochloride (Tris-HCl), 0.4 % (w/v) sodium dodecyl sulfate (SDS), pH 8.8

Final volume	250 mL	500 mL	1000 mL
Tris-HCL	45.43 g	90.86 g	181.7 g
0.4% SDS	1.0 g	2.0 g	4.0 g

Stacking gel buffer: 0.5 M Tris-HCl, 0.4 % (w/v) SDS, pH 6.8

Final Volume	250 mL	500 mL
Tris-HCL	15.14 g	30.28 g
0.4% SDS	1 g	2 g

Acrylamide solution: Ultra-Pure Protogel containing 30% acrylamide. Stored away from light and at room temperature (National Diagnostics; Atlanta, US).

Ammonium persulphate: 100 $mg \cdot mL^{-1}$, prepared freshly for each gel

Electrophoresis running buffer (10x): 1 x buffer is 0.025 M Tris-HCl, 0.1 %: (w/v) SDS, 0.2 M glycine, pH 8.3

Final volume	2.5 L (10 x stock)
Tris-HCL	75.75 g
0.1% SDS	25 g
Glycine	360 g

SDS-transfer buffer: pH 8.3 (for a volume of 2L mix with 1.6 L distilled water)

Tris (48 mM)	11.63 g
Glycine (39 mM)	5.89 g
SDS	0.75 g (final percentage 0.0375%)
Methanol	400 mL

Tris-buffered saline buffer **TBS and TBS-T:** 0.09% NaCl, 1M Tris-HCl, pH 7.4 containing Tween-20 at a final concentration of 0.1% (v/v) (TBS-T).

CHAPTER 3 - BREAKFAST-EXERCISE & GLYCAEMIA

Pre-Exercise Breakfast Ingestion *versus* Extended Overnight Fasting Increases Postprandial Glucose Flux after Exercise in Healthy Men

Edinburgh, R. M., Hengist, A., Smith, H. A., Travers, R. L., Koumanov, F., Betts, J. A., Thompson, D., Walhin, J-P., Wallis, G.A., Hamilton, L.D., Stevenson, E. J., Tipton, K.D. & Gonzalez, J.T. (2018). Pre-exercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. *American Journal of Physiology-Endocrinology and Metabolism, 315 (5),* 1062-1074.

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Acknowledgements:

The authors thank the volunteers for their time and effort in participating. We also thank Sewa Abdullah for assistance with Mass Spectrometry analysis.

Sources of Support:

The European Society for Clinical Nutrition and Metabolism and The Rank Prize Funds (both to J. T. Gonzalez). F. Koumanov is funded by the Medical Research Council (MR/P002927/1).

Trial Registration: https://clinicaltrials.gov/ [Number: NCT02258399]

Data Access Statement: The complete data set relating to this manuscript can be found at: https://researchdata.bath.ac.uk/608/

RELEVANCE TO THESIS

Requiring participants to fast overnight before laboratory-based exercise trials is common procedure in an attempt to control for their metabolic status. However, this standardisation may preclude the application of findings to situations most representative of normal daily living for people living in developed countries. For many humans, exercise is regularly performed in the evening (261) and in concert with the high carbohydrate diet that is typically consumed in developed countries (186), it is likely that the majority of exercise sessions are performed after eating carbohydrates rather than in a fasted state. We also know that feeding before exercise alters the glycaemic responses to exercise. Indeed, blood glucose fluxes during exercise (272) and blood glucose concentrations after exercise (15) can be increased if carbohydrate rich meals are consumed before exercise. However, as discussed in **Chapter 1**, the mechanisms underpinning differences in glycemia at post-exercise meals with altered pre-exercise feeding are not currently clear.

Characterising blood glucose flux at meals consumed after exercise performed in an overnight fasted state compared to after breakfast is important. This will provide information regarding whether results from studies of exercise in a fasted-state can be applied to exercise more representative of normal daily living and will advance our current understanding of post-exercise metabolism. Secondly, investigating glycemic responses to food ingestion provides insights into mortality risk (276). Given that most eating occasions in developed countries take place whilst still in a postprandial state from previous meal(s), it is important to assess postprandial responses to sequential meal consumption. Any glucose lowering effect at post-exercise meals with exercise performed during morning fasting compared to after prior meal consumption may help to protect against the risk of T2D and CVD (276) and risk of macro- and microvascular complications in people with T2D (278) with a repeated exposure to this stimulus. Some adaptations to nutrient-exercise manipulations have also been suggested to be due to repeated transient acute responses to exercise (426, 427). Therefore, characterising acute responses to nutrient-exercise manipulations was the aim of **Chapter 3**.

This declaration concerns the article entitled:			
Pre-Exercise Breakfast Ingestion *versus* Extended Overnight Fasting Increases Postprandial Glucose Flux after Exercise in Healthy Men			
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]			
Publication details (reference)	Edinburgh, R. M., Hengist, A., Smith, H. A., Travers, R. L., Koumanov, F., Betts, J. A., Thompson, D., Walhin, J-P., Wallis, G.A., Hamilton, L.D., Stevenson, E. J., Tipton, K.D. & Gonzalez, J.T. (2018). Pre-exercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. *American Journal of Physiology-Endocrinology and Metabolism, 315 (5),* 1062-1074.		
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Candidate's contribution to the paper (provide details, and also indicate as a percentage)	**Formulation of ideas:** JTG, KDT, DLH, EJS, JAB and DT designed the research **Design of methodology:** JTG, KDT, DLH, EJS, JAB and DT predominantly designed the methodology. **Experimental work:** RME (> 80 %), JTG, AH, HS, RLT and J-PW conducted the research and RME, JTG, AH, HS, FK, and GAW analysed the data. RME was present for the data collection on trial days, prepared and analysed the blood samples (for metabolites and hormones) and muscle samples (western blotting), completed data and statistical analysis and was heavily involved in all aspects of the research. RME completed the preparation and analysis of blood samples for glucose enrichments at the University of Birmingham (Sewa Abdullah assisted with Mass Spectrometry analysis). **Presentation of data in journal format:** RME (> 80 %) and JTG wrote the paper, and all authors contributed to earlier versions of the manuscript and approved the final version of the manuscript.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	R M Edinburgh	**Date**	2nd July 2019

3089

ABSTRACT

Aims: To characterize postprandial glucose flux after exercise in the fed *versus* overnight fasted-state and to investigate potential underlying mechanisms. **Methods:** In a randomized order, twelve men underwent breakfast-rest (BR; 3 h semi-recumbent), breakfast-exercise (BE; 2 h semi-recumbent before 60-min of cycling (50% peak power output) and overnight fasted-exercise (FE; as per BE omitting breakfast) trials. An oral glucose tolerance test (OGTT) was completed post-exercise (post-rest on BR). Dual stable isotope tracers ([U-^{13}C] glucose ingestion and [6,6-$^{2}H_2$] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signalling, respectively. Plasma intestinal fatty acid binding (I-FABP) concentrations were determined as a marker of intestinal damage. **Results:** Breakfast before exercise increased post-exercise plasma glucose disposal rates during the OGTT, from 44 g·120 min^{-1} in FE [35 to 53 g·120 min^{-1}] (mean [normalized 95% CI]) to 73 g·120 min^{-1} in BE [55 to 90 g·120 min^{-1}; p = 0.01]. This higher plasma glucose disposal rate was, however, offset by increased plasma glucose appearance rates (principally OGTT-derived), resulting in a glycemic response that did not differ between BE and FE (p = 0.11). Plasma I-FABP concentrations during exercise were 264 pg·mL^{-1} [196 to 332 pg·mL^{-1}] lower in BE *versus* FE (p = 0.01). **Conclusion:** Breakfast before exercise increases post-exercise postprandial plasma glucose disposal, which is offset (primarily) by increased appearance rates of orally-ingested glucose. Therefore, metabolic responses to fed-state exercise cannot be readily inferred from studies conducted in a fasted state.

INTRODUCTION

Postprandial glycemia is a strong predictor of future mortality and morbidity. Even in people without diabetes, those with greater blood glucose excursions after feeding are at an increased risk of cardiovascular disease (47, 48). This glycemic response to food ingestion is dictated by blood glucose kinetics (i.e. the balance between the rates of glucose appearance into blood and glucose disposal from blood into peripheral tissues). Exercise potently increases glucose disposal from the blood into skeletal muscle (52), and regular exercise is therefore recommended as a lifestyle strategy to improve glycemic control.

Habitual responses to exercise and nutrition are however, the culmination of not only chronic adaptations, but also the acute effects of each exposure to these daily behaviors (5, 6, 22). For example, each bout of exercise potently stimulates post-exercise insulin sensitivity and muscle glucose uptake (52). However, despite increases in blood glucose disposal rates, endurance-type exercise does not always reduce postprandial glucose excursions in the post-exercise period (20, 54). The finding that postprandial blood glucose concentrations are not lowered post-exercise is because when exercise is performed (at least in the fasted state), the increase in postprandial blood glucose disposal after exercise can be offset - *and even superseded* - by increases in both endogenous and meal-derived blood glucose appearance rates (34, 54).

Whilst fasting prior to laboratory trials is common in order to control for baseline metabolic status, these conditions may preclude the application of findings to situations most representative of daily living. For example, most people living in developed countries spend the majority of a typical day in the postprandial state (13, 55). Therefore, most eating occasions and exercise sessions will take place in the context of this postprandial situation (23). It has previously been shown by others that plasma glucose fluxes *during* exercise (16), and by us that plasma glucose *concentrations* after exercise (24), are elevated by pre-exercise feeding. However, the effect of prior feeding on *post-exercise* plasma glucose flux has never been assessed. Therefore, there is a distinct lack of understanding

regarding postprandial glucose kinetics under scenarios that are most representative of daily living, and it may not be valid to generalize existing observations of exercise in the fasted state. Moreover, an understanding of the underlying mechanisms responsible for any differences in postprandial glucose flux post-exercise, with prior feeding *versus* fasting, is still required.

This study therefore aimed to characterize postprandial plasma glucose kinetics after: 1) breakfast and rest; 2) breakfast and exercise and 3) overnight fasted-state exercise, while also exploring potential mechanisms (intramuscular signaling and markers of intestinal damage) to explain any differences in glucose flux between these conditions.

METHODS

Ethical Approval

All trials were undertaken at the University of Bath (Bath, UK) in accordance with the Declaration of Helsinki. The study was approved by the National Health Service South-West Research Ethics Committee (reference: 15/SW/0006) and registered at clinicaltrials.gov as NCT02258399. Written, informed consent was obtained from all participants prior to their participation.

Study Design

This study was a randomized cross-over design (randomization performed by JTG with Research Randomizer version 3.0, http://www.randomizer.org/). Preliminary testing was followed by three trials (separated by > 7 d), namely, breakfast-rest (BR), breakfast-exercise (BE) and overnight fasted-exercise (FE). A schematic for the study protocol is shown in **Figure 1**. For all trials participants arrived at the laboratory after a 12 to 14-h overnight fast. In BR, a porridge breakfast was consumed, followed by 3 h of rest, and then a 2-h oral glucose tolerance test (OGTT). In BE, the same breakfast was consumed, before 2 h rest and 60 min of cycling, prior to the OGTT. In FE, breakfast was omitted but the trial otherwise replicated BE. By necessity of design (food intake/exercise) the intervention was open label. Within-lab testing conditions were not different

across the trials ([mean ± SD] ambient temperature [23.7 ± 0.5 °C on BR, 23.7 ± 0.6 °C on BE, 23.6 ± 0.7 °C on FE) and barometric pressure [734 ± 5 mmHg on BR, 736 ± 6 mmHg on BE, 736 ± 5 mmHg on FE]; all $p > 0.05$).

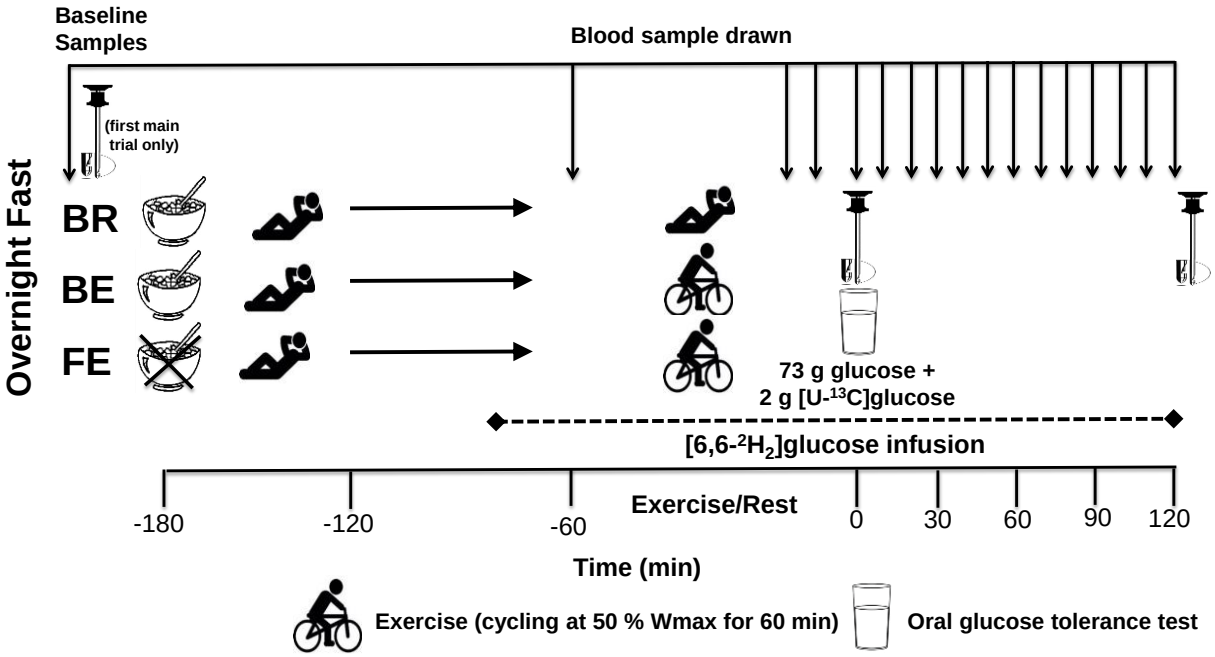


Figure 1. Schematic. An oral glucose tolerance test was conducted after breakfast followed by rest (BR), breakfast followed by exercise (BE), or extended overnight fasting followed by exercise (FE). Dual stable isotope tracers ([U-^{13}C] glucose ingestion and [6,6-$^{2}H_2$] glucose infusion) and muscle biopsies were used to assess postprandial plasma glucose kinetics and intramuscular signaling.

Participants

Twelve healthy and physically active men (self-reported as regular exercisers engaging in at least 30 min of exercise a minimum of 3 times per week) were recruited from Bath and North East Somerset, between May and November 2015. Participant characteristics are shown in **Table 1**. Exclusion criteria included any history of metabolic disease, or condition that may have posed undue personal risk to the participant or introduced bias to the study.

Table 1. Participant Characteristics

Age (y)	23 (3)
Stature (cm)	179.8 (4.4)
Body mass (kg)	76.3 (7.9)
Body Mass Index ($kg \cdot m^{-2}$)	23.6 (2.90)
Fat mass (kg)[1]	10.6 (3.7)
Fat Mass Index ($kg \cdot m^{-2}$)[1]	3.26 (1.12)
Body fat (%)[1]	14 (4)
Fat free mass (kg)[1]	65.5 (6.4)
$\dot{V}O_2$peak ($L \cdot min^{-1}$) *	4.00 (0.72)
$\dot{V}O_2$peak ($mL \cdot kg \cdot min^{-1}$) *	53 (10)
Peak Power Output (W)	317 (67)
HR_{MAX}, ($beats \cdot min^{-1}$)	189 (10)

Data are presented as means and (standard deviation). $\dot{V}O_2$peak = peak oxygen uptake. *n* = 12. **n* = 11, due to technical difficulties with the breath-by-breath analysis during one participant's preliminary testing. [1] = derived by dual-energy x-ray absorptiometry (DEXA).

Preliminary Testing

Participants were asked to refrain from strenuous physical activity for 24 h prior to preliminary testing, but were asked to otherwise maintain their normal physical activity behaviors. They abstained from alcoholic and caffeinated drinks for 24 h prior to this visit. Food intake ceased at 8 pm on the evening before testing and participants fasted overnight (minimum 12 h), consuming only water (*ad libitum*) during this period. In addition, they were asked to consume 568 mL of water at least 1 h prior to testing, and to void immediately prior to arriving at the laboratory. Upon arrival, the participant's stature was measured (Frankfurt plane) to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK). Body mass was recorded to the nearest 0.1 kg (only light clothing permitted) using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). A whole-body dual energy x-ray absorptiometry scan was completed to quantify fat and fat-free mass

(DEXA; Discovery, Hologic, Bedford, UK). Participants performed an incremental exercise test at a self-selected cadence on an electronically-braked ergometer (Excalibur Sport, Lode Lode® Groningen, Netherlands). They were permitted to adjust the saddle and handlebar heights to their preferred position, which were replicated for cycling during the exercise trials. The initial exercise intensity was 50 W and this was increased by 50 W every four min, for four stages. Thereafter, the intensity was increased by 20 W every min until volitional exhaustion. Heart rate (Polar Electro Oy, Kempele, Finland) and continuous breath-by-breath measurements were recorded throughout (TrueOne2400, ParvoMedics, Sandy, USA). Volume and gas analyzers were calibrated with a 3 L calibration syringe (Hans Rudolph, Kansas City, USA) and a calibration gas (balance nitrogen mix; 16.04% O_2, 5.06% CO_2; BOC Industrial Gases, Linde AG, Munich, Germany), respectively. Peak power output (PPO) was calculated as the work rate of the last completed stage, plus the fraction of time spent in the final non-completed stage, multiplied by the work rate increment. Peak oxygen uptake ($\dot{V}O_2$ peak) was calculated as the highest average $\dot{V}O_2$ over a rolling 30-s period.

Main Trials

Participants refrained from strenuous physical activity, alcohol and caffeine for 24 h before all trials. They recorded the composition of their evening meal on the day before the first main trial and replicated this meal for subsequent trials, in accordance with procedures for standardizing postprandial glucose tolerance testing (10). This pre-trial standardisation protocol has been previously shown to be effective at producing overnight-fasted muscle glycogen concentrations, liver glycogen concentrations and intramyocellular lipid that are standardised across multiple trial days, in a similar population (21). To help ensure physical activity standardization, participants completed a physical activity diary and wore a physical activity monitor (Actiheart^{TM}; Cambridge Neurotechnology, Papworth, UK) for 24 h before all trials (pre-trial 24-h physical activity energy expenditure [(mean ± standard deviation) 988 ± 500 kcal on BR, 1022 ± 521 kcal on BE, 992 ± 313 kcal on FE; all p >0.05; n = 9]. Participants arrived at the laboratory at 0800 ± 1 h following a 12 to 14-h overnight fast and this arrival time was replicated for

the subsequent trials. They were asked to void and all further urine samples were collected for the remainder of the trial to allow for urinary nitrogen excretion to be estimated from urine urea concentrations. Participants then placed their dominant hand into a heated-air box (Mass Spectrometry Facility; The University of Vermont & University of Vermont Medical Center, Burlington, USA) set to 55 °C. After 20 min of rest, an intravenous catheter (BD Venflon Pro, BD, Helsingborg, Sweden) was fitted into a heated dorsal hand vein (retrograde) and a 10-mL baseline blood sample was drawn, before a 5-min expired gas sample was collected. On the first main trial for each participant (see **Figure 1**), a baseline muscle sample was taken from the *vastus lateralis* to allow for an assessment of pathways involved in exercise and insulin signaling in muscle [5' AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), protein kinase B (herein referred to as Akt2), and Akt substrate of 160 kDa (AS160).

In BE and BR, a porridge breakfast was then consumed within 10 min (3 h pre-OGTT) and in FE participants were allowed water only. The breakfast was 72 g of instant refined oats (Oatso Simple Golden Syrup, Quaker Oats) and 360 mL of semi-skimmed milk (Tesco), providing 431 kcal of energy ([1803 kJ]; 65 g CHO, 11 g FAT, 19 g PRO). The breakfast was high-carbohydrate (57% of energy intake) and high glycemic-index [oatmeal, made from mix (Quaker Oats) has a glycemic index of 69 (17)], as is commonly consumed in developed countries. Due to the co-ingestion of milk, this breakfast would produce a high insulinemic response (38, 46). Breakfast consumption (or omission on FE) was followed by 2 h of rest, where participants remained in a semi-recumbent position, completing resting activities (e.g. watching television), with expired gas samples collected every 60 min. After 1 h 40 min of rest, (1 h 20 min pre-OGTT) a catheter was inserted into an antecubital vein (the contralateral arm to the one used for blood sampling). A primed infusion of [6,6-$^{2}H_{2}$]glucose was initiated and continued for the remaining within-lab component of the trial (Cambridge Isotope Laboratories, MA, USA; prime: 13.5 µmol.kg^{-1}; infusion: 0.35 µmol.kg^{-1}.min^{-1}). After 20 min (60 min pre-OGTT), and on BE and FE only, participants began 60 min of cycling at 50% PPO on an ergometer (Lode Corival, Lode B.V, Groningen, Netherlands).

The cadence was self-selected (replicated for both exercise trials) and the power output was monitored via a computerized system. In BR, participants remained rested in the semi-recumbent position during this period. Expired gas samples were collected every 15 min and blood samples were collected at 40 and 50 min of exercise (20 and 10 min pre-OGTT). Immediately post-exercise (or post-rest in BR) a muscle sample was taken from the *vastus lateralis*. Then a 2-h OGTT was completed, with arterialized blood sampled at 10-min intervals and expired gas sampled every 60 min. The OGTT was 73 g of glucose (81 g of dextrose monohydrate corrected for water content; Myprotein, Northwich, UK) and 2 g of [U-^{13}C]glucose (99%; Cambridge Isotope Laboratories, MA, USA), to allow the rate of appearance of the orally ingested glucose (Ra_{OGTT}) to be assessed. A final muscle sample was taken post-OGTT (OGTT 120 min).

Tracer approach, blood sampling and analysis

A dual-tracer approach was employed, where the tracer infusion rate was doubled during exercise (on BE and FE) to account for an expected increase in endogenous glucose production (1) and reduced to 80% of baseline at OGTT 20 min (all trials) to account for an expected suppression of endogenous glucose production after oral glucose ingestion (9). This approach reduces changes in the tracer-to-tracee ratio, thereby permitting more accurate estimations of glucose kinetics (4). Arterialized blood was sampled from a heated dorsal hand vein at baseline, at 60-min intervals during the initial 2-h rest period, at 40 and 50 min of the exercise period (or post-rest in BR) and at 10-min intervals during the OGTT. Whole blood was dispensed into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) which were first centrifuged (4 °C and 3500 *g*) for 10-min (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK) to obtain plasma. The plasma was then dispensed into 0.5 mL aliquots and immediately frozen at -20 °C, before longer-term storage at -80 °C.

Plasma glucose (intra-assay coefficient of variation [CV], 3.2%; inter-assay CV 3.8%), lactate (intra-assay CV, 1.0%; inter-assay CV 4.8%), and triglyceride (intra-assay CV, 1.4%; inter-assay CV 4.0%) concentrations were measured

using an automated analyzer (Daytona; Randox Lab, Crumlin, UK) as per the manufacturer's instructions. Plasma insulin concentrations were measured using a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden; intra-assay CV, 5.7%; inter-assay CV 9.9%). Plasma intestinal fatty acid binding protein (I-FABP) concentrations were measured as a marker of intestinal cell damage using a commercially available ELISA kit (Hycult Biotech; intra-assay CV, 6.0%). Plasma NEFA concentrations were determined using an enzymatic colorimetric assay (WAKO Diagnostics; intra-assay CV, 8.9%; inter-assay CV 10.4%). For all of these analyses, all plasma samples were analyzed in batch after all sample collection was completed, and for a given participant all samples (from the three trials) were run on the same plate.

Plasma [U-^{13}C]glucose and [$^{2}H_2$]glucose enrichments were determined by gas chromatography-mass spectrometry (GC-MS: GC, Agilent 6890N; MS, Agilent 5973N; Agilent Technologies, Stockport, UK). Plasma glucose was extracted using methanol-chloroform and hydrochloric acid, dried under nitrogen gas, and then derivatised using the heptofluorobuyric acid method as previously described (30). The glucose derivative was acquired by selected ion monitoring at mass-to-charge ratios (*m*/*z*) 519, 521 and 525 for [^{12}C], [6,6-$^{2}H_2$]- and [U-^{13}C]-glucose, respectively. Glucose enrichments of [^{13}C] and [$^{2}H_2$] in plasma were determined using standard curves for [^{13}C] and [$^{2}H_2$] glucose, and enrichments were expressed relative to those at 519 (M+0). The baseline sample was used for every trial to account for background isotopic plasma enrichments. To reduce any impact of analytical variability on calculations of glucose kinetics, glucose and enrichment data were curve fitted as previously described (63).

Muscle sampling and analysis

Muscle samples were collected from the *vastus lateralis* under local anesthesia (~5 mL of 1% lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples were taken from a 3-5-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction (57). Samples were immediately extracted from the needle and frozen in liquid nitrogen, before

longer-term storage at -80 °C. The order of dominant or non-dominant leg was counterbalanced across trials for the OGTT 0 min and the 120 min samples. Samples were taken from separate skin incision sites, with these > 2 cm proximal to any previous incision on the same leg (59). For the OGTT 0 min sample (post exercise [or rest in BR]) the incision was made prior to cycling (BE and FE) and closed with Steristrips, to allow for an immediate sample post-exercise.

Frozen wet tissue (20-30 mg) was freeze-dried, powdered, and dissected free of visible blood and connective tissue and added to ice cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Sodium deoxycholate; 0.1% SDS and 0.1% NP-40] with a protease (Thermo Scientific) and phosphatase inhibitor cocktail (Millipore). Samples were homogenized with a dounce homogenizer (~ 40 passes), incubated (60 min at 4 °C with rotation) and centrifuged for 10 min (4 °C and 20,000 *g*). The protein content of the resultant supernatant was measured via a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). For Western blots, an equal amount of protein (40 µg) was loaded per lane for each sample and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine SDS–polyacrylamide gels (7% for p-AMPK^{Thr172}, total AMPK, p-ACC^{Ser79} and total ACC, 10% for p-Akt^{Ser473} and total Akt2 and 8% for p-Akt^{Thr308}, p-AS160^{Thr642} and total AS160). Gels were electro-blotted using a semi-dry transfer onto a nitrocellulose membrane. Membranes were washed in Tris-buffered saline (0.09% NaCl, 100 mM Tris–HCl pH 7.4) with 0.1% Tween 20 (TBS-T) and incubated for 30-min in a blocking solution (5% non-fat dry milk in TBS-T; Marvel, Premier International Foods Ltd, UK). The membranes were incubated overnight at 4 °C with primary antibodies against p-AMPK^{Thr172}, p-ACC^{Ser79}, p-Akt^{Ser473}, p-Akt^{Thr308} and p-AS160^{Thr642} (Cell Signaling Technologies, USA). In the morning, membranes were washed in TBS-T and incubated with a 1:4000 dilution of anti-species IgG horseradish peroxidase-conjugated secondary antibodies made up in the aforementioned blocking solution. After further washes, membranes were incubated in an enhanced chemiluminescence (ECL) reagent and visualized using a chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, USA). Nitrocellulose membranes were then incubated for 30-min

at 50 °C in a stripping solution [62.5 mM Tris pH 6.7, 2%SDS, 100 mM 2-mercaptoethanol], before re-blotting for total AMPK, total ACC, total Akt2 (Cell Signaling Technologies, USA) and total AS160 (Merck-Milliopore, UK). For these analyses, all samples from each participant (all three trials) were run on the same gel. Band densities were quantified using VisionWorksLS Image Acquisition and Analysis Software for Windows (UVP, Upland, USA). For all of the signalling molecules reported in this experiment a ratio of phosphorylated to total protein was calculated and the results were expressed relative to the baseline sample.

Substrate utilization

Expired gas samples were collected at baseline, during the initial 2-h rest period and the OGTT at 60-min intervals (for 5 min), and at 15-min intervals (for 1 min) during the exercise period (or rest in BR). For all samples, participants were provided with the mouthpiece 1 min before gas collections for a stabilization period. Samples were collected in 200 L Douglas bags (Hans Rudolph, Kansas City, USA) through falconia tubing (Baxter, Woodhouse and Taylor Ltd, Macclesfield, UK). Concurrent measures of inspired air were made to correct for changes in ambient O_2 and CO_2 concentrations (7). Expired O_2 and CO_2 concentrations were measured in a known volume of each sample, using paramagnetic and infrared transducers respectively (Mini HF 5200, Servomex Group Ltd., Crowborough, East Sussex, UK). The sensor was calibrated using concentrations of low (99.998% Nitrogen, 0% O_2 and CO_2) and high (balance nitrogen mix, 16.04% O_2, 5.06% CO_2) calibration gases (both BOC Industrial Gases, Linde AG, Munich, Germany). Urinary nitrogen excretion was estimated from urine urea concentrations, which were measured on an automated analyzer (Daytona; Randox Lab, Crumlin, UK), to allow for protein oxidation to be accounted for in calculations of substrate utilization rates.

Calculations and statistical analysis

A sample size estimation was completed *a priori* with the total rate of plasma glucose appearance as the primary outcome measure. Rose *et al. (54)* reported a difference in the total plasma glucose appearance of (mean ± SD) 1600 ± 1300

μmol·kg^{-1} during an OGTT after rest *versus* after fasted exercise. Using this effect size, and an alpha level of 0.05, we calculated that 12 participants were required for an 80% probability of statistically detecting an effect in the Ra_{TOTAL} using a crossover design with three trials and a two-tailed, one-way ANOVA.

The total and incremental area underneath the concentration-time curve (AUC or iAUC, respectively) for each plasma metabolites or hormones was calculated using the trapezoid rule. The AUC or iAUC for each plasma metabolite or hormone was then divided by either the duration of the total within-lab period (300-min) or the OGTT observation period (120-min), as appropriate, to provide a time-averaged value (mmol·L^{-1}), which are used as summary measures. Plasma glucose and insulin concentrations during the OGTT were used to estimate insulin sensitivity (ISI) according to the equation of Matsuda (41): [FPG and FPI are fasting plasma glucose and insulin concentrations, and MPG and MPI are mean plasma glucose and insulin concentrations in the OGTT (41)]:

$$ISI_{MATSUDA}(au) = \frac{10{,}000}{\sqrt{FPG(mgdL^{-1}) \bullet FPI(mIUmL^{-1}) \bullet MPG(mgdL^{-1}) \bullet MPI(mIUmL^{-1})}}$$

Plasma glucose kinetics were determined using Radziuk's two-compartment non-steady state model (50, 51) and SAMM II software (SAAM II v2.3, The Epilson Group, Charlottesville, VA, USA). This model reduces errors in estimations of glucose kinetics that are apparent when using Steele's (56) one compartment model (53). The total rate of plasma glucose appearance (Ra_{TOTAL}) and glucose disappearance (Rd) were calculated as follows:

Equation 1:

$$Ra_{TOTAL}(t) = \frac{F}{E_1(t)} - \frac{V_1 \cdot G(t)}{E_1(t)} \cdot \dot{E}_1(t) + k_{12}\left(\frac{q_2^{iv}(t)}{E_1(t)} - Q_2(t)\right)$$

Equation 2:

$$Rd\ (t) = Ra_{TOTAL} - V_1 \cdot \dot{G}(t) - k_{21} \cdot V_1 \cdot G(t) + k_{12} \cdot Q_2(t)$$

Where F is the [6,6-$^{2}H_2$] infusion, V_1 is the glucose volume of distribution [4% of body mass (kg)], $E_1(t)$ the [$^{2}H_2$] plasma glucose enrichment (mole percent excess) at time *t*, $\dot{E}_1$ (t) the change in E over time [derivate of E], G*(t)* the plasma glucose concentrations at time t, $\dot{G}(t)$ the change in G over time [derivate of G], k_{12} and k_{21} are fixed rate constants between the peripheral and the accessible compartments (0.05 min^{-1} and 0.07 min^{-1} respectively) and q_2^{iv} and Q_2 are the amounts of the tracer [$^{2}H_2$] and tracee in the peripheral compartment respectively, evaluated by integrating the two-compartment model.

The [U-^{13}C] enrichment of the orally ingested glucose and the Ra_{TOTAL} (from *Equation 1*) were used to calculate the plasma rate of appearance of glucose from the OGTT (Ra_{OGTT}). In these equations, r_1 is the ratio of the infusion [$^{2}H_2$] and oral [U-^{13}C] glucose tracer concentrations in plasma, $\dot{r}$ (*t*) is the change in r over time [derivate of r], g is the [U-^{13}C] glucose tracer in plasma, q_o^{iv} is the amount of the [U-^{13}C] tracer in the peripheral compartment (by integrating the two-compartment model), and E_{OGTT} is the [U-^{13}C] enrichment of the OGTT.

Equation 3:

$$ra^{O}(t) = Ra_{TOTAL}(t)\frac{F}{r_1(t)} - \frac{V_1 \cdot g(t)}{r_1(t)} \cdot \dot{r}_1(t) + k_{12}\left[\frac{q_2^{iv}(t)}{r_1(t)} - q_2^{0}(t)\right]$$

Equation 4:

$$Ra_{OGTT}(t) = ra^{O}(t)\left[\frac{1}{E_{OGTT}}\right]$$

The metabolic clearance rate was calculated as the Rd divided by the plasma glucose concentrations for a given time point (G_1)

Equation 5:

$$\text{Metabolic Clearance Rate} = Rd(t) / G_1$$

Rates of whole-body fat and carbohydrate utilization were calculated using the expired gas samples and stoichiometric equations (31). Adjustments were made to account for the contribution made by the oxidation of protein (estimated via

urinary urea nitrogen). Plasma glucose utilization was assumed to be equivalent to the plasma glucose rate of disappearance (Rd) as has been confirmed previously (32). Muscle glycogen utilization during exercise (BE and FE only) was calculated as total carbohydrate utilization during exercise minus plasma glucose utilization during exercise. Due to these methods, this estimate of muscle glycogen utilization will include the utilization of other non-glucose carbohydrates (e.g. lactate). Both the production and utilization of ketone bodies can influence the respiratory exchange ratio and therefore theoretically complicate the estimates of carbohydrate oxidation during exercise. However, during short-duration, moderate intensity exercise this effect is negligible (31). Within-lab, energy expenditure was determined assuming that lipids, glucose and glycogen give 9.42, 3.74 and 4.15 $kcal \cdot g^{-1}$ respectively (31).

One-way, repeated measures ANOVA were used to assess differences between trials at baseline and for summary measures (e.g. AUCs). If multiple comparisons were necessary, two-way repeated measures ANOVAs (time x trial) was used to identify differences between trials. Degrees of freedom for *F* values were Greenhouse-Geisser corrected for epsilon < 0.75, with Huynh-Feldt corrections used for less severe asphericity. If time x trial interaction effects were identified, multiple paired *t*-tests were used to locate variance, with Holm-Bonferroni step-wise adjustments to control for inflated type I errors. Pearson *r* and Spearman *R* were used to explore correlations between variables displaying normal and non-normal distribution, respectively. Unless otherwise stated, data in text, figures and tables are means ± 95% confidence intervals, which were normalized by removing between-subject variance (presented as 95% nCI) (39). All statistical analyses were completed using IBM SPSS statistics version 22 for windows (IBM, New York, USA), with the exception of the Holm-Bonferroni step-wise adjustments and the calculation of normalized confidence intervals which were completed using Microsoft Excel [2013]. Graph Pad Prism 7 software (La Jolla, CA, USA) was used for preparation of the manuscript figures. A complete set of muscle samples was only obtained from nine participants. Due to cannulation difficulties, for one participant's BR trial the last blood sample obtained was at

OGTT 70 min and for a different participant's BE trial the last sample was at OGTT 60 min. For these trials (2 of 36) the group average was used for the missing data. Sensitivity analysis was completed for all measures involving blood samples and including/excluding these two participants did not influence any of the primary outcome measures. For clarity, the *n* is presented in all legends.

RESULTS

Plasma glucose kinetics

The plasma glucose disappearance rate (Rd) displayed a time x trial interaction (F = 3.123, p = 0.05), whereby plasma glucose Rd was higher during exercise *versus* rest (**Figure 2A**). Compared to extended overnight fasting, breakfast ingestion prior to exercise further increased the plasma glucose Rd during and post-exercise (i.e. during the OGTT; **Figure 2A**). A main effect of trial was detected for the plasma glucose Rd during the OGTT (F = 7.079, p = 0.01), whereby the Rd was 45 g·120 min^{-1} in BR [95% nCI: 36 to 62 g·120 min^{-1}] *versus* 73 g·120 min^{-1} in BE ([95% nCI: 55 to 90 g·120 min$^{-1;}$ p = 0.09 *versus* BR) and 44 g·120 min^{-1} in FE [95% nCI: 35 to 53 g·120 min^{-1}]; p = 0.01 *versus* BE). Metabolic clearance rates showed a main effect of trial, with the highest rates also apparent in BE (**Figure 2B**; F = 7.849, p < 0.01 *versus* BR and FE). A main effect of trial was detected for Ra_{TOTAL} during the OGTT ([g·120 min^{-1}]; F = 3.915, p = 0.05) which was highest in BE (**Figure 2C)**. However, after post-hoc adjustment the difference between trials was less apparent (p = 0.19 for BE *versus* BR and p = 0.09 for BE *versus* FE). A similar pattern was observed for the rate of appearance of glucose from the OGTT in plasma (Ra_{OGTT}) and a trial x time interaction was detected (**Figure 2D**; F = 3.134, p = 0.04). A main effect of trial was detected for the total Ra_{OGTT} (F = 5.915, p = 0.02), which was 49 g·120 min^{-1} in BE [95% nCI: 44 to 53 g·120 min^{-1}] (65% of the OGTT [59 to 71%]) *versus* 42 g·120 min^{-1} in BR [95% nCI: 36 to 46 g·120 min^{-1}] (56% of the OGTT [50 to 62%]; p = 0.11 *versus* BE) and 41 g·120 min^{-1} in FE [95% nCI: 35 to 47 g] (55% of the OGTT [49 to 61%] p = 0.06 *versus* BE). The plasma enrichment of [$^{2}H_{2}$] - and [^{13}C]-glucose are shown in **Figures 2E** and **2F**, respectively.

Plasma glucose concentrations

No difference between trials was detected for plasma glucose concentrations at baseline (Figure 3A; p > 0.05). Thereafter, a trial x time interaction was apparent (F = 2.957, p = 0.01). During the exercise period (rest in BR), plasma glucose concentrations were higher in BR *versus* BE at 40 min, and in BR *versus* FE at 50 min (both p < 0.05). At OGTT 0 min, glucose concentrations were higher in BR *versus* BE, and during the OGTT they were initially higher in BR *versus* BE and in BR *versus* FE (all p < 0.05), but no further differences were then detected (Figure 3A). Peak plasma glucose concentrations were higher in BR *versus* BE (p = 0.03), but not different in BE *versus* FE (Table 2; p > 0.05). A main effect of trial was detected for the within-lab (300-min) glucose AUC which was higher in BR *versus* BE (Table 2; p = 0.05). However, no main effect of trial was detected for the OGTT (120-min) iAUC (Figure 4A; F = 2.524, p = 0.11).

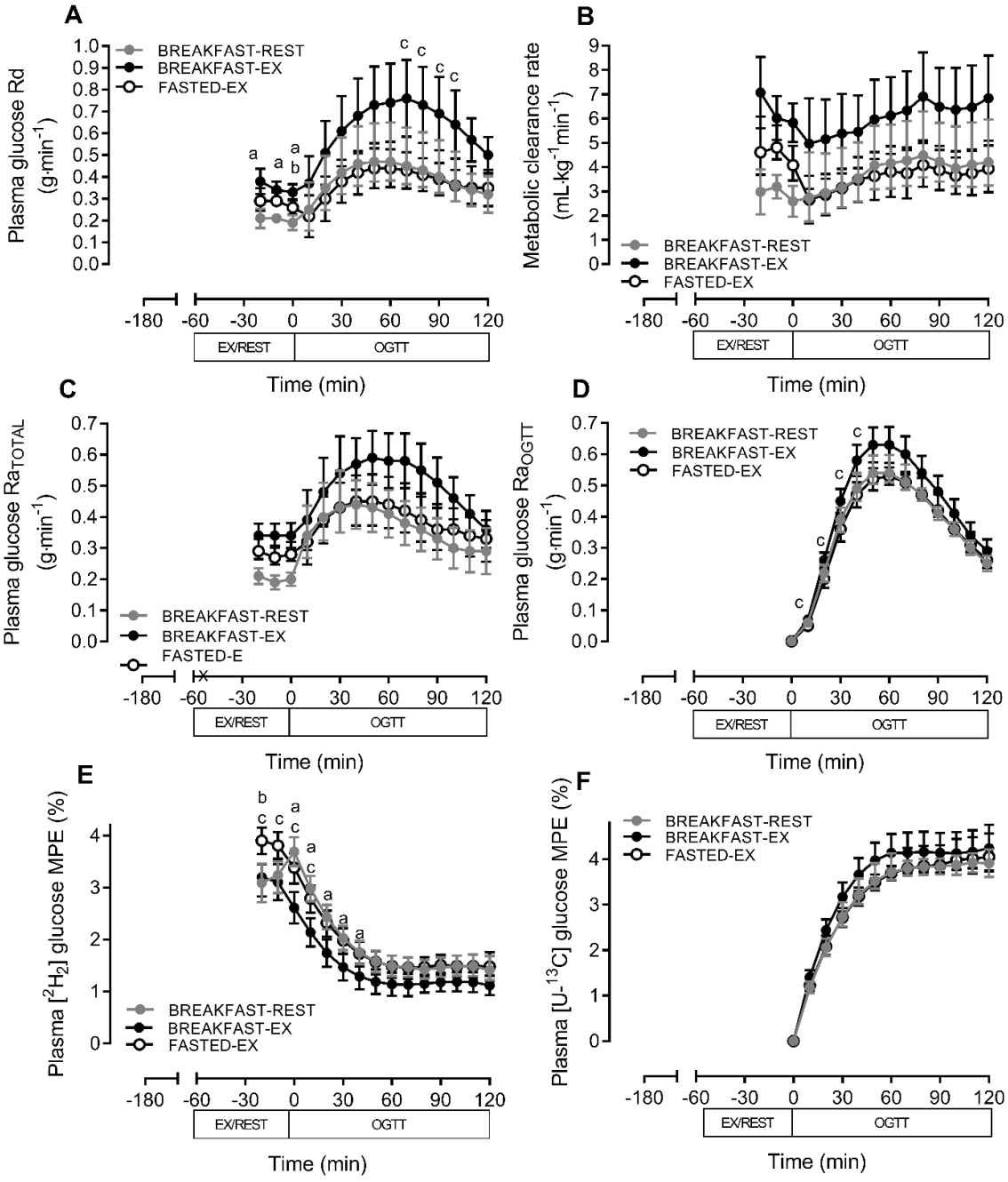
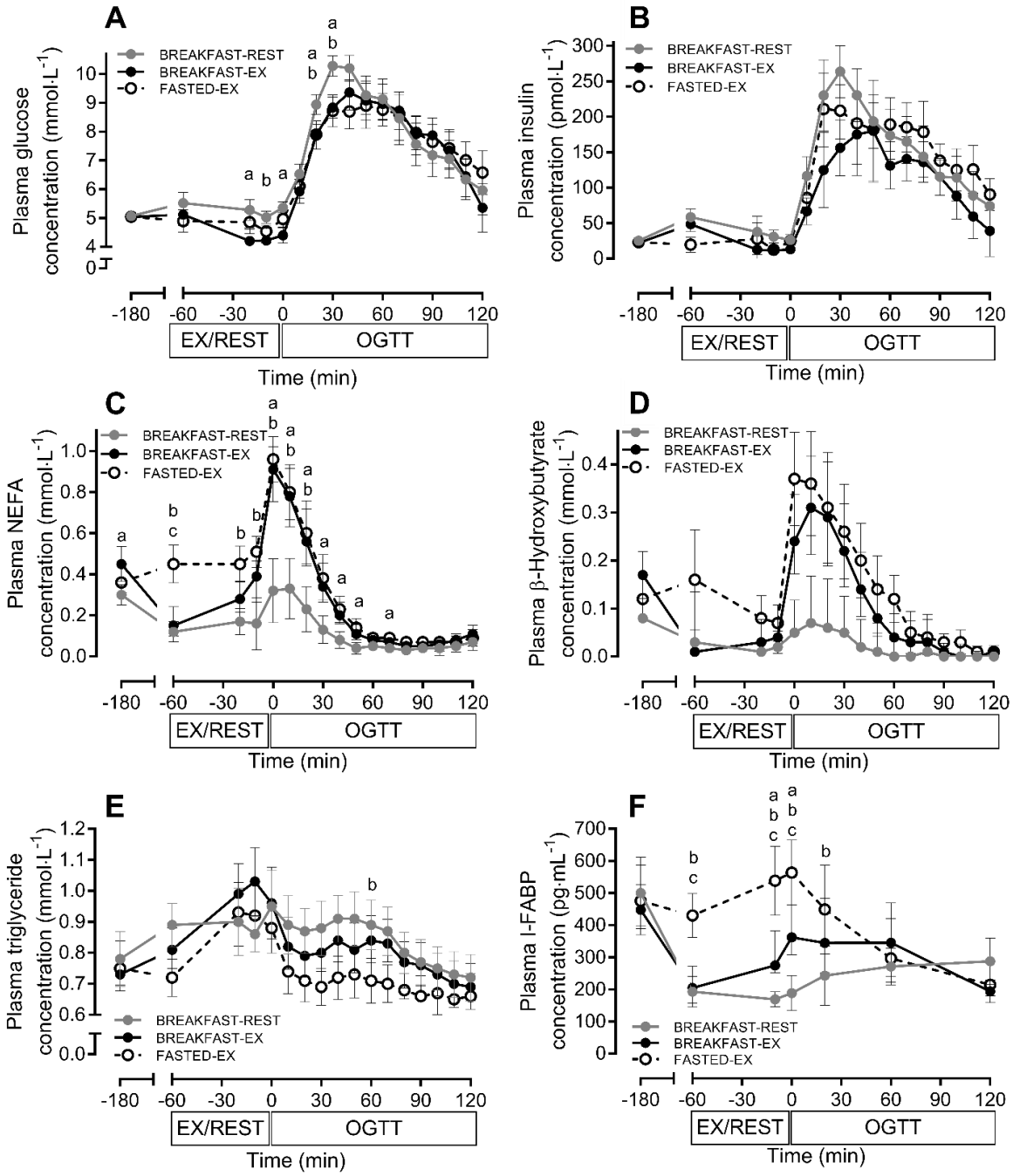


Figure 2. Plasma glucose disposal rate (Rd) (**A**), metabolic clearance rate (**B**), the total rate of plasma glucose appearance (Ra_{TOTAL}) (**C**), the rate of appearance of glucose in plasma from the oral glucose tolerance test (Ra_{OGTT}) (**D**), and the plasma enrichments of [$^{2}H_{2}$]-glucose (**E**) and [^{13}C]-glucose (**F**) before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. [a] = difference between breakfast rest *versus* breakfast exercise; [b] = breakfast rest *versus* fasted exercise and [c] = breakfast exercise *versus* fasted exercise with $p < 0.05$.



3547
3548 **Figure 3.** Plasma glucose (**A**), plasma insulin (**B**), plasma non-esterified fatty
3549 acids (NEFA; **C**), plasma β-hydroxybuturate (**D**) plasma triglyceride (**E**), and
3550 plasma intestinal fatty acid binding protein (IFAB-P; **F**) concentrations before and
3551 during an oral glucose tolerance test that was conducted after breakfast followed
3552 by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX),
3553 or extended overnight fasting followed by exercise (FASTED-EX). Data are
3554 means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference
3555 between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus*
3556 fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

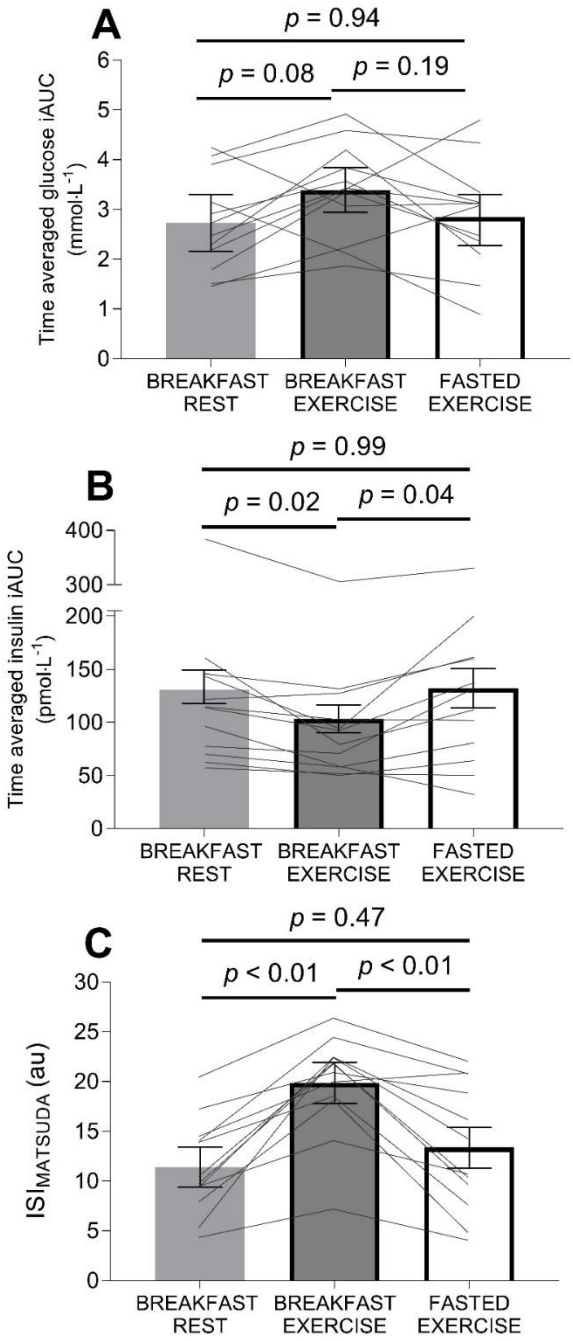


Figure 4. The time-averaged (120-min) plasma glucose (**A**) and plasma insulin (**B**) incremental area under the curves (iAUC) and the Matsuda insulin sensitivity index (**C**; $ISI_{MATSUDA}$) for an oral glucose tolerance test conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals, with individual data shown as grey lines. *n* = 12 healthy men.

Plasma insulin concentrations

At baseline, there was no difference between trials for plasma insulin concentrations (**Figure 3B**; $p > 0.05$). Main effects of time ($F = 4.351$, $p < 0.01$) and trial ($F = 7.986$, $p < 0.01$) were detected, but there was no trial x time interaction effect ($F = 2.395$, $p = 0.07$). Peak (and time to peak) plasma insulin concentrations are shown in **Table 2**. A main effect of trial was detected for the within-lab (300-min) insulin AUC which was higher in BR *versus* BE ($p < 0.01$), but not different in BE *versus* FE (**Table 2**; $p = 0.10$). A main effect of trial was apparent for the insulin OGTT iAUC [**Figure 4B**; (120-min) $F = 5.132$, $p = 0.02$] which was lower in BE *versus* BR [by 27.34 pmol·L^{-1} (95% nCI: 12.10 to 45.80 pmol·L^{-1}); $p = 0.02$] and lower in BE *versus* FE [by 28.67 pmol·L^{-1} (95% nCI: 10.21 to 47.12 pmol·L^{-1}); $p = 0.04$]. There was a main effect of trial for the $ISI_{MATSUDA}$ insulin sensitivity index (**Figure 4C**; $F = 22.790$, $p < 0.01$), which was higher in BE *versus* BR [by 8.45 au, (95% nCI: 6.42 to 10.47 au); $p < 0.01$] and in BE *versus* FE [by 6.49 au (95% nCI: 2.93 to 8.51 au); $p < 0.01$].

Plasma non-esterified fatty acid (NEFA) concentrations

A main effect of trial ($F = 4.314$, $p = 0.04$) was detected for plasma NEFA at baseline, with concentrations of 0.30 mmol·L^{-1} in BR (95% nCI: 0.25 to 0.35 mmol·L^{-1}), 0.45 mmol·L^{-1} in BE (95% nCI: 0.36 to 0.53 mmol·L^{-1}; $p = 0.03$ BE *versus* BR) and 0.36 mmol·L^{-1} in FE (95% nCI: 0.31 to 0.44 mmol·L^{-1}; $p = 0.12$ FE *versus* BR and BE). Thereafter, a time x trial interaction effect was apparent (**Figure 3C**; $F = 11.438$, $p < 0.01$), where plasma NEFA concentrations were lowered by breakfast consumption in BR and BE, and remained lower during the exercise in BE *versus* FE, before increasing during the initial OGTT period in BE and FE *versus* BR. A main effect of trial was detected for the total within-lab plasma NEFA (300-min) AUC and the NEFA OGTT (120-min) AUC which in both instances was lower in BR *versus* BE and FE (**Table 2**; all $p < 0.01$).

Other plasma metabolites

No differences were detected between trials at baseline for plasma β-hydroxybuturate concentrations (**Figure 3D;** $p > 0.05$). Thereafter, a time x trial

interaction effect was apparent (F = 6.310, p < 0.01) where concentrations were lowered by breakfast in BR and BE. Plasma β-hydroxybuturate concentrations remained lower during exercise in BE *versus* FE, but increased during the OGTT with BE and FE *versus* BR. However, with post-hoc adjustment, the differences between trials for plasma β-hydroxybuturate concentrations became less clear (all p > 0.05). The within-lab (300-min) β-hydroxybuturate AUC was lower with BR *versus* BE (p = 0.03), but did not differ in BE and FE (**Table 2**; p = 0.35). No baseline differences were detected for triglyceride concentrations (**Figure 3E**, p > 0.05) but a time x trial interaction effect was apparent (F = 3.994, p < 0.01). There was an effect of trial for the within-lab (300-min) and OGTT (120-min) triglyceride AUC, which tended to be lower in FE *versus* BE, but with post-hoc adjustment this difference between trials was less clear (**Table 2;** p > 0.05). Plasma lactate concentrations at baseline were not different across trials (p > 0.05) but were lower in BR *versus* BE and FE in the exercise period (rest in BR) and at OGTT 0 min, but were then higher in BR *versus* BE during the OGTT (time x trial; F = 20.305, p < 0.01). No effect of trial was detected for the total within-lab (300-min) lactate AUC, but a main effect of trial was detected for the lactate OGTT (120-min) AUC, which higher in BR *versus* BE (**Table 2;** p < 0.01).

Plasma intestinal fatty acid binding protein (I-FABP) concentrations

There was no difference between trials at baseline for plasma I-FABP concentrations (**Figure 3F;** p > 0.05), but these were lower after breakfast (time x trial interaction effect; F = 6.844, p < 0.01) in BR and BE *versus* FE (both p < 0.05). During and post-exercise (or rest in BR), I-FABP concentrations were lower in BR and BE *versus* FE and remained lower in BR *versus* FE until OGTT 20 min (all p < 0.05). The within-lab (300-min) I-FABP AUC was lower in BR and BE *versus* FE (**Table 2**; p = 0.01 and p = 0.05 respectively).

Activation of exercise and insulin signalling pathways in skeletal muscle

Time x trial interaction effects were apparent for $AMPK^{Thr172}$ (ratio p-AMPK to total-AMPK) and ACC^{Ser79} (ratio pACC to total ACC) phosphorylation, if normalized to the baseline muscle sample (**Figure 5A**; F = 5.154, p = 0.04 and

Figure 5B, $F = 5.881$, $p = 0.02$, respectively). Compared to the breakfast-rest trial (BR), skeletal muscle AMPK^{Thr172} phosphorylation was higher post-exercise (or post-rest in BR) in the breakfast and exercise (BE) trial [by 1.9 fold (95% nCI: 0.9 to 2.8 fold); $p = 0.04$] and was also higher in BE *versus* the fasted-exercise (FE) trial [by 1.0 fold (95% nCI: 0.2 to 2.0 fold); $p = 0.01$]. A similar pattern was apparent for ACC phosphorylation, which was higher post-exercise (or post-rest in BR) in BE *versus* BR [by 6.7 fold (95% nCI: 5.4 to 8.0 fold); $p = 0.03$] but did not differ between BE and FE ($p = 0.09$). By OGTT 120 min, ACC^{Ser79} and AMPK^{Thr172} phosphorylation had returned to baseline levels in all three trials (all $p > 0.05$). No time x trial interaction ($F = 2.110$, $p = 0.16$) nor a main effect of trial ($F = 0.098$, $p = 0.83$) was detected for Akt^{Ser473} (ratio p-Akt^{Ser473} to total-Akt2) phosphorylation (**Figure 6A**). A main effect of time ($F = 9.907$, $p = 0.01$) was observed, where Akt^{Ser473} phosphorylation was elevated at OGTT 120 min in all trials. Similarly no time x trial interaction ($F = 1.533$, $p = 0.25$) nor a main effect of trial ($F = 0.484$, $p = 0.56$) was detected for Akt^{Thr308} (ratio p-Akt^{Thr308} to total-Akt2) phosphorylation (**Figure 6B**). A main effect of time ($F = 10.598$, $p = 0.01$) was also detected for this phosphorylation site, whereby Akt^{Thr308} phosphorylation was elevated at OGTT 120 min in all trials. For AS160^{Thr642} phosphorylation (ratio p-AS160^{Thr642} to total-AS160), a time x trial interaction was detected (**Figure 6C**; $F = 4.430$, $p = 0.03$), where the AS160^{Thr642} phosphorylation was not different with BR and BE at any time, was higher pre-OGTT in BE compared to FE ($p = 0.04$), but was not different between BE and FE at 120-min post-OGTT ($p = 0.69$).

Table 2. Peak plasma concentrations, time to peak concentrations, and the time-averaged area under the curve (AUC) for various metabolites and hormones measured during the total within-lab period (300-min) and/or during an oral glucose tolerance test observation (120-min) that was conducted after breakfast followed by rest (Breakfast-Rest), breakfast followed by exercise (Breakfast-Exercise), or extended overnight fasting followed by exercise (Fasted-Exercise).

	Breakfast Rest	**Breakfast Exercise**	**Fasted Exercise**	
Peak glucose conc. (mmol·L ^{1})	10.62 (9.98, 11.25)	9.65 (9.27, 10.03) ^{a}	9.82 (9.44, 10.45)	p=0.03
Time to peak glucose (min)	**36 (24, 47)**	**49 (36, 62)**	**49 (36, 61)**	***p*=0.12**
Glucose AUC_{TOTAL} (mmol·L^{-1})	6.41 (6.21, 6.60)	6.05 (5.90, 6.15) ^{a}	6.07 (5.93, 6.27)	p=0.01
Peak insulin conc. (pmol·L^{-1})	**286 (231, 341)**	**209 (148, 269)**	**282 (222, 337)**	***p*=0.07**
Time to peak insulin (min)	38 (26, 49)	56 (47, 65) ^{a}	43 (33, 54)	p=0.03
Insulin AUC_{TOTAL} (pmol·L^{-1})	**88 (79, 97)**	**62 (54, 71) ^{aa}**	**76 (67, 85)**	***p*<0.01**
NEFA AUC_{TOTAL}(mmol·L^{-1})	0.16 (0.12, 0.19)	0.28 (0.24, 0.33)^{aa}	0.37 (0.31,0.42)^{bb}	p<0.01
NEFA AUC_{OGTT} (mmol·L^{-1})	**0.10 (0.06, 0.14)**	**0.24 (0.20, 20.29) ^{aa}**	**0.26 (0.22, 0.31) ^{bb}**	***p*<0.01**
Triglyceride AUC_{TOTAL} (mmol·L^{-1})	0.85 (0.79, 0.91)	0.81 (0.75, 0.86)	0.75 (0.70, 0.79)	p=0.03
Triglyceride AUC_{OGTT} (mmol·L^{-1})	**0.84 (0.77, 0.92)**	**0.79 (0.72, 0.86)**	**0.70 (0.64, 0.77)**	***p*=0.02**
Lactate AUC_{TOTAL} (mmol·L^{-1})	1.05 (0.96, 1.13)	1.19 (1.08, 1.30)	1.16 (1.07, 1.27)	p=0.06
Lactate AUC_{OGTT} (mmol·L^{-1})	**1.12 (1.04, 1.20)**	**0.97 (0.89, 1.04) ^{aa}**	**1.03 (0.96, 1.11)**	***p*=0.02**
β-hydroxybuturate AUC (mmol·L^{-1})	0.03 (0.00, 0.06)	0.08 (0.06, 0.13) ^{a}	0.14 (0.09, 0.19) ^{b}	p=0.03
I-FABP AUC_{TOTAL} (pg·mL^{-1})	**279 (242, 317)**	**304 (267, 366)**	**415 (353, 476) $^{b\ cc}$**	***p*<0.01**

Data are means and (normalized 95% confidence intervals). conc. = concentration; AUC_{TOTAL} = the time-averaged area underneath the concentration-time curve for the total within-lab period (300-min); AUC_{OGTT} = the time-averaged area underneath the concentration-time curve for the oral glucose tolerance test (120-min); I-FABP = intestinal fatty acid binding protein. n = 12 healthy men ^{a} represents a difference between breakfast rest and breakfast exercise, ^{b} a difference between breakfast rest and fasted exercise and ^{c} a difference between breakfast exercise and fasted exercise with $p < 0.05$. ^{aa}, ^{bb} or ^{cc} is with $p < 0.01$.

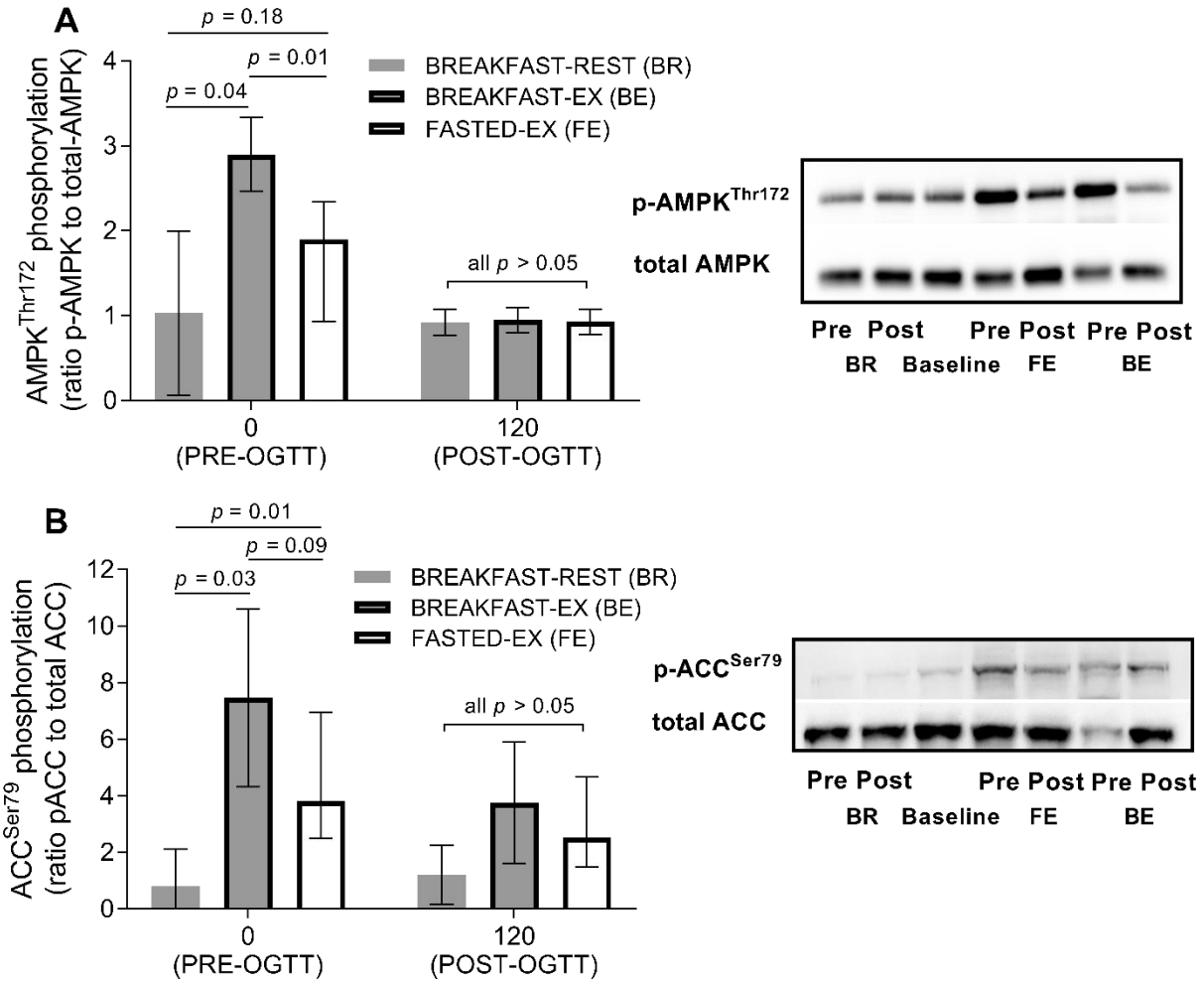


Figure 5. The phosphorylation of 5' AMP-activated protein kinase (**A**; phospho AMPK^{Thr172}, ratio p-AMPK to total-AMPK) and the phosphorylation of acetyl-CoA carboxylase (**B**; phospho ACC^{Ser79}, ratio p-ACC to total-ACC) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

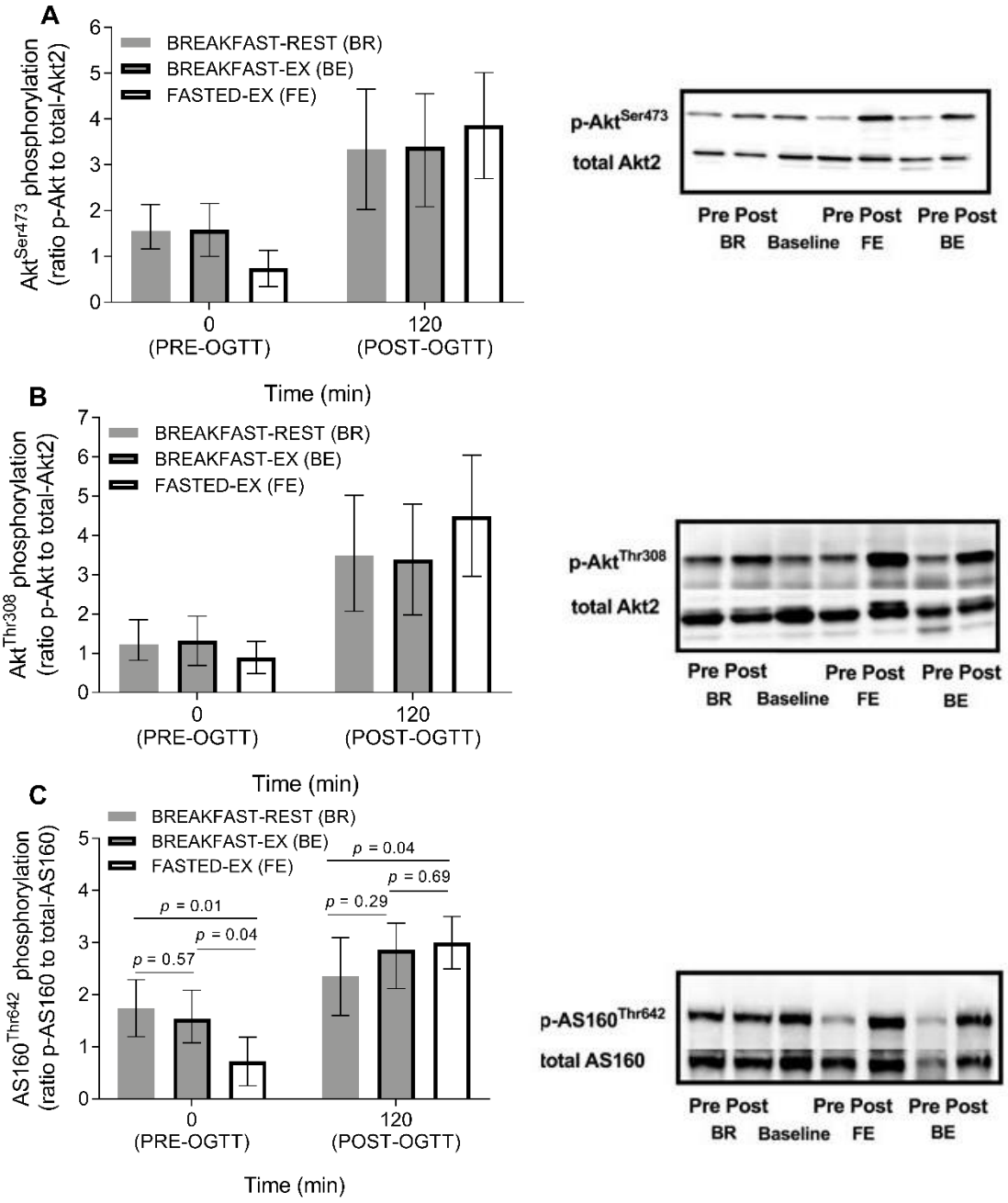


Figure 6. The phosphorylation of Akt2 (**A**; phospho Akt^{Ser473}, ratio p-Akt^{Ser473} to total-Akt2 and **B**; phosphor Akt^{Thr308}, ratio p-Akt^{Thr308} to total-Akt2) and the phosphorylation of AS160 (**C**; $AS160^{Thr642}$, ratio p-$AS160^{Thr642}$ to total-AS160) before (PRE-OGTT) and after (POST-OGTT) an OGTT that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

Substrate utilization

Across the duration of the trial carbohydrate utilization was higher in BE *versus* BR (**Figure 7**; by 514 kcal, [95% nCI: 452 to 576 kcal] and higher in BE *versus* FE (by 124 kcal, [95% nCI: 18 to 230 kcal], both $p < 0.01$). This difference in carbohydrate utilization between BE and FE was derived from a higher utilization of plasma glucose *and* other carbohydrate sources (i.e. muscle glycogen, but also plasma lactate) in BE ($p = 0.02$ and $p = 0.04$ respectively). Within-lab fat utilization did not differ between BR and BE ($p = 0.25$), but was higher in FE *versus* BE (by 138 kcal, [95% nCI: -6 to 224 kcal], $p = 0.03$). Muscle glycogen utilization during exercise (g·kg body mass^{-1}) was positively correlated ($R = 0.64$, $p < 0.01$) with skeletal muscle ACC^{Ser79} phosphorylation (ratio p-ACC to total-ACC) after exercise conducted following breakfast (BE) or overnight fasting (FE).

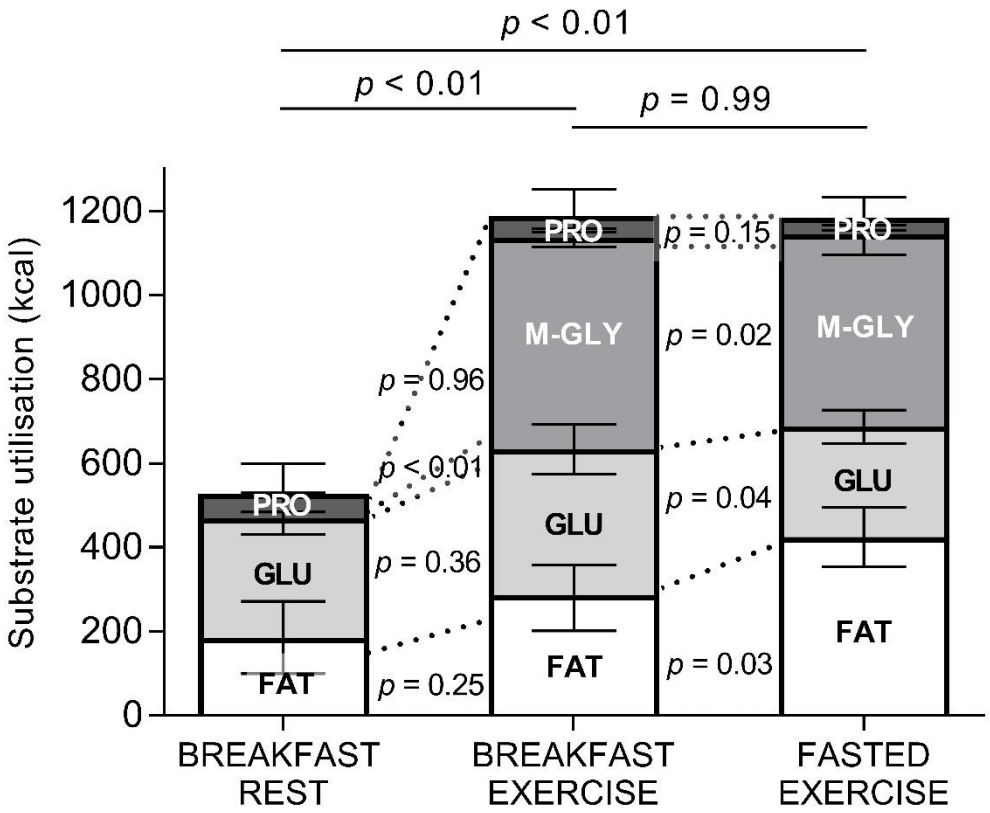


Figure 7. Substrate utilization after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Within-lab substrate utilization was calculated assuming that the plasma glucose disappearance rate was equivalent to plasma glucose utilization. Glycogen utilization during exercise (on breakfast exercise and fasted exercise only) was calculated as total carbohydrate utilization (from indirect calorimetry) minus plasma glucose utilization during exercise. This estimate of muscle glycogen utilization will therefore include the utilization of other non-glucose (e.g. lactate) carbohydrates. GLU = glucose; M-GLY = muscle glycogen; PRO = protein. Data are presented as means ± normalized 95% confidence intervals. *n* = 12 healthy men.

DISCUSSION

This is the first study to assess the effect of pre-exercise feeding on postprandial plasma glucose kinetics after exercise. Our data demonstrate that pre-exercise feeding increases plasma glucose disposal during meals consumed after exercise, despite lower insulinemia in this condition. Characterizing glucose flux at meals is important because this determines postprandial glycemia; a predictor of cardiovascular disease risk (47, 48). Previous work has only studied postprandial glucose flux after fasted-state exercise. As most people consume food and perform exercise while still in a postprandial period from a prior meal (13, 23, 55) our results describe the physiological responses to feeding that are more readily applicable to scenarios that are representative of normal daily living. Moreover, our data demonstrate that metabolic responses to exercise conducted in an overnight fasted state and to meals that are consumed post-exercise cannot be easily extrapolated to conditions where breakfast has been consumed.

The disposal of plasma glucose (the disappearance rate) into skeletal muscle is elevated after exercise, via insulin-dependent and -independent pathways (25). We observed a higher postprandial plasma glucose disposal rate with breakfast *versus* fasting before exercise. The higher postprandial plasma glucose rate of disposal with pre-exercise breakfast ingestion was apparent despite lower insulinemia in the breakfast-exercise trial. At rest, breakfast consumption improves glucose tolerance and insulin sensitivity at subsequent meals [known as the “second-meal effect” (5, 20, 26)]. Mechanisms likely relate to delayed gastric emptying (19), a potentiation of early phase insulinemia at the second meal (37) and enhanced glucose uptake into muscle due to increased GLUT4 trafficking (18). Our findings show that breakfast ingestion (*versus* fasting) prior to exercise enhances subsequent glucose disposal at post-exercise meals in the presence of lower insulinemia, suggesting that the second-meal effect is maintained even if exercise is performed between meals. Whilst the effects of pre-exercise feeding on the metabolic responses during subsequent exercise is well characterised (15, 43), our data therefore provide new insights regarding postprandial glucose metabolism after exercise in the fed *versus* fasted state.

Molecular insulin-signaling pathways are instrumental mediators of glucose disposal in response to exercise and/or nutrition (11). We therefore determined the activation status of key proteins involved in glucose uptake in skeletal muscle [the primary site of postprandial glucose disposal (14)]. Akt activation (Thr^{308} and Ser^{473} phosphorylation) two hours after OGTT began, was unaffected by prior exercise or prior breakfast ingestion. However, the timing of muscle sampling could be responsible for this result. It is possible that differences in Akt activation between trials may have been apparent earlier in the postprandial period, as peak Akt phosphorylation can be variable, occurring as early as 30 min after an OGTT (35). Thus, despite the lack of a measurable difference, we cannot rule out a role for insulin signaling in the glucose disposal responses we observed. Distal proteins within the insulin signalling pathway can be activated after exercise, without detectable differences in Akt phosphorylation (61). We therefore measured $AS160^{Thr642}$ activation as this phosphorylation site has been previously shown to be activated by both insulin *and* exercise stimulation (58). However, our data show that $AS160^{Thr642}$ phosphorylation was not different two hours post-OGTT with breakfast *versus* fasting before exercise, providing further evidence that differences in early stages of the activation of the insulin signalling pathway were not responsible for the higher plasma glucose disposal rate we observed when breakfast was ingested before exercise.

AMPK activity also plays a key role in muscle glucose uptake and stimulates GLUT4 translocation (27). The greater post-exercise skeletal muscle AMPK activation with breakfast prior to exercise may have contributed to the higher glucose disposal rate in that trial. This AMPK response seems to be specific to skeletal muscle, as we have previously shown that post-exercise adipose tissue AMPK content is unaffected by pre-exercise feeding (12). The increase in skeletal muscle AMPK activity with pre-exercise feeding that we report in the present study may seem surprising given that the ingestion of large amounts (> 200 g) of carbohydrate before *and* during exercise can blunt AMPK signaling in muscle (3, 29). This blunting is partly because low muscle glycogen concentrations stimulate AMPK activity (42). The *modest amount* (65 g) of carbohydrate ingested by

participants in our study may explain why we did not observe an elevated AMPK response in our fasted-exercise trial. For example, when smaller carbohydrate doses are ingested before and/or during exercise (~ 120 g or less) the exercise induced increase in the phosphorylation of AMPK and ACC is not always suppressed compared to when a placebo is ingested (2, 36, 58), although in one study this result was apparent despite a suppression of α2-AMPK activity when carbohydrate was ingested (2).

The heightened AMPK response observed in the current study with breakfast before exercise may be explained by the *type* of carbohydrate ingested before exercise (high-glycemic index) in the breakfast-exercise trial, as this can stimulate muscle glycogen use during exercise, especially when no carbohydrate is ingested during the activity (62). Thus, the high-glycemic index breakfast with a modest carbohydrate content in the present study, may have stimulated muscle glycogen utilization during exercise without supplying sufficient carbohydrate to replace additional glycogen utilization, resulting in lower post-exercise muscle glycogen concentrations with breakfast *versus* fasting before exercise. This could explain the enhanced exercise-induced AMPK response if breakfast was consumed prior to exercise. However, we had insufficient tissue to measure muscle glycogen *concentrations in all participants,* so our data only provide rates of muscle glycogen *utilization (which was higher with feeding versus fasting before exercise)*. Nevertheless, in the two participants for whom we had sufficient tissue to perform glycogen analyses, these data agreed with the tracer-derived calculations of muscle glycogen utilization (data not shown) and AMPK signaling via western blotting. Consistent with this, the present data demonstrated that post-exercise ACC^{Ser79} phosphorylation – as a marker of activation of the AMPK pathway - positively correlated with muscle glycogen utilization during exercise. Thus, together our results suggest that the increased post-exercise plasma glucose disposal rates with pre-exercise feeding *versus* fasting may have been mediated through enhanced AMPK signaling, which is itself a consequence of altered fuel use during exercise. AMPK signaling and resulting muscle glycogen concentrations following exercise after breakfast should now be explored.

The higher plasma glucose disposal rate (despite lower plasma insulin concentrations), that we observed in the breakfast-exercise trial, may be due to differences in GLUT4 trafficking downstream of the signalling proteins we measured. Insulin-stimulated GLUT4 translocation and insulin sensitivity of skeletal muscle are increased *in vitro*, if muscle is pre-treated with insulin, without differences in Akt^{Ser473} or Akt^{Thr308} activation, or the transport activity of GLUT4 (18). This suggests priming of skeletal muscle by prior insulin exposure enhances subsequent insulin action. Pre-treatment with insulin *and* exercise augments this response, possibly because insulin (33) and exercise (28) stimulate GLUT4 translocation from different intracellular stores (8, 49). As such, in this work, prior breakfast *and* exercise (the multiple stimuli in BE), may have enhanced skeletal muscle GLUT4 translocation during the OGTT. Although technically challenging future work should now quantify GLUT4 trafficking with fed *versus* fasted state exercise to confirm this. It is also possible that hepatic glucose disposal accounts for some of the increase in glucose disposal following BE *versus* FE. Similarly to skeletal muscle, insulin-stimulated hepatic glucose uptake is enhanced by prior exposure to insulin in dogs (44, 45). Thus, assuming that this response persists in humans and after exercise, priming of the liver by prior breakfast could also contribute to greater glucose disposal during meals consumed post-exercise.

The increases in post-exercise glucose disposal with prior breakfast consumption were, however, offset by alterations in plasma glucose appearance. As such, postprandial glucose *concentrations* did not differ between the two exercise trials. Postprandial plasma glucose appearance rates are determined by three main factors: 1) the appearance of glucose from the meal; 2) residual appearance of glucose from previous meals; and/or 3) liver glucose output (glycogenolysis or gluconeogenesis). We showed that alterations in the postprandial plasma glucose appearance rate (Ra_{TOTAL}) after exercise with prior breakfast was mostly driven by increased appearance of glucose from the post-exercise OGTT. These alterations in the Ra_{TOTAL} suggest that differences in gut function (i.e. increased intestinal damage or absorptive capacity) and/or splanchnic blood flow altered the Ra_{TOTAL}. Thus, breakfast prior to exercise may alter postprandial glycemia via

factors related to intestinal absorption and splanchnic handling of glucose, rather than just glucose metabolism by skeletal muscle.

There are several potential mechanisms which may explain the differences in glucose appearance rates due to intestinal absorption and/or splanchnic handling of glucose. Plasma I-FABP concentrations are used as a marker of damage to intestinal epithelial cells (60). We noted lower plasma I-FABP concentrations in the breakfast-exercise *versus* fasted-exercise trial, despite increased plasma glucose appearance rates of the orally-ingested glucose post-exercise. It is therefore unlikely that increased intestinal damage was responsible for the higher plasma glucose appearance rates we observed with feeding *versus* fasting before exercise. The better maintenance of splanchnic perfusion during exercise with prior feeding is a likely explanation for this response (15). If splanchnic perfusion was better maintained during exercise with prior feeding, this may have also *directly* facilitated higher OGTT-derived glucose appearance rates in that trial *versus* the fasted-exercise trial. It should be acknowledged however, that this intestinal damage response may be specific to cycling, and could differ with other exercise modalities (e.g. running), within the context of pre-exercise feeding. An alternative mechanism for higher appearance rate of orally-ingested glucose with feeding before exercise could also be that apical glucose transporters were primed by the prior breakfast ingestion (40). Although further underlying mechanism(s) remain unclear, and should therefore be investigated with future work, we showed that a major determinant of post-exercise glycemia (plasma glucose appearance rates) are altered by pre-exercise feeding and that this is unlikely to be explained by increases in intestinal damage.

Our results show that the metabolic and intramuscular signaling responses to exercise conducted in a fed state cannot be readily inferred from responses observed with exercise in a fasted state. As well as a continual investigation of the mechanisms responsible for differences in postprandial glucose metabolism with altered pre-exercise feeding, future work should study whether the results we observed are apparent with different post-exercise meals (including the co-

ingestion of fat and protein). Moreover, if the acute alterations in postprandial metabolism translate into longer-term differences in insulin sensitivity with repeated bouts of exercise in the fed *versus* fasted state and in populations with obesity, should now be a focus for future work.

To conclude, eating breakfast (*versus* fasting) before exercise increases post-exercise plasma glucose disposal rates but this is offset by increases in appearance rates of (mainly) orally-ingested glucose, a result that does not appear to be explained by a greater intestinal damage response to the exercise. We showed that pre-exercise breakfast consumption lowers insulinema at meals that are consumed post-exercise, providing new evidence that the second meal effect is maintained even when exercise is performed between eating occasions.

REFERENCES FOR CHAPTER 3

1. Ahlborg G, Felig P, Hagenfeldt L, Hendler R, and Wahren J. Substrate turnover during prolonged exercise in man: splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *Journal of Clinical Investigation* 53: 1080, 1974.
2. Akerstrom TC, Birk JB, Klein DK, Erikstrup C, Plomgaard P, Pedersen BK, and Wojtaszewski JF. Oral glucose ingestion attenuates exercise-induced activation of 5′-AMP-activated protein kinase in human skeletal muscle. *Biochemical and biophysical research communications* 342: 949-955, 2006.
3. Bartlett JD, Louhelainen J, Iqbal Z, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B, and Morton JP. Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 304: 450-458, 2013.
4. Basu R, Di Camillo B, Toffolo G, Basu A, Shah P, Vella A, Rizza R, and Cobelli C. Use of a novel triple-tracer approach to assess postprandial glucose metabolism. *American Journal of Physiology-Endocrinology And Metabolism* 284: E55-E69, 2003.
5. Betts JA, Chowdhury EA, Gonzalez JT, Richardson JD, Tsintzas K, and Thompson D. Is breakfast the most important meal of the day? *Proceedings of the Nutrition Society* 75: 1-11, 2016.
6. Betts JA, Richardson JD, Chowdhury EA, Holman GD, Tsintzas K, and Thompson D. The causal role of breakfast in energy balance and health: a randomized controlled trial in lean adults. *Am J Clin Nutr* 100: 539-547, 2014.
7. Betts JA, and Thompson D. Thinking outside the bag (not necessarily outside the lab). *Medicine and Science in Sports and Exercise* 44: 2040, 2012.
8. Bradley H, Shaw CS, Bendtsen C, Worthington PL, Wilson OJ, Strauss JA, Wallis GA, Turner AM, and Wagenmakers AJ. Visualization and quantitation of GLUT4 translocation in human skeletal muscle following glucose ingestion and exercise. *Physiological reports* 3: 2015.
9. Breen L, Philp A, Shaw CS, Jeukendrup AE, Baar K, and Tipton KD. Beneficial effects of resistance exercise on glycemic control are not further improved by protein ingestion. *PLoS One* 6: e20613, 2011.
10. Brouns F, Bjorck I, Frayn K, Gibbs A, Lang V, Slama G, and Wolever T. Glycaemic index methodology. *Nutrition research reviews* 18: 145, 2005.
11. Cartee GD. Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. *Diabetologia* 58: 19-30, 2015.
12. Chen YC, Travers RL, Walhin JP, Gonzalez JT, Koumanov F, Betts JA, and Thompson D. Feeding Influences Adipose Tissue Responses to Exercise in Overweight Men. *Am J Physiol Endocrinol Metab* 313: ajpendo 00006 02017, 2017.
13. de Castro JM. The time of day of food intake influences overall intake in humans. *The Journal of nutrition* 134: 104-111, 2004.
14. DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, and Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *Journal of Clinical Investigation* 76: 149-155, 1985.
15. Enevoldsen L, Simonsen L, Macdonald I, and Bülow J. The combined effects of exercise and food intake on adipose tissue and splanchnic metabolism. *The Journal of Physiology* 561: 871-882, 2004.
16. Febbraio MA, Keenan J, Angus DJ, Campbell SE, and Garnham AP. Preexercise carbohydrate ingestion, glucose kinetics, and muscle glycogen use: effect of the glycemic index. *Journal of Applied Physiology* 89: 1845-1851, 2000.
17. Foster-Powell K, Holt SHA, and Brand-Miller JC. International table of glycemic index and glycemic load values: 2002. *The American Journal of Clinical Nutrition* 76: 5-56, 2002.

18. Geiger PC, Han DH, Wright DC, and Holloszy JO. How muscle insulin sensitivity is regulated: testing of a hypothesis. *American Journal of Physiology-Endocrinology and Metabolism* 291: E1258-E1263, 2006.
19. Gentilcore D, Chaikomin R, Jones KL, Russo A, Feinle-Bisset C, Wishart JM, Rayner CK, and Horowitz M. Effects of fat on gastric emptying of and the glycemic, insulin, and incretin responses to a carbohydrate meal in type 2 diabetes. *The Journal of Clinical Endocrinology & Metabolism* 91: 2062-2067, 2006.
20. Gonzalez JT. Paradoxical second-meal phenomenon in the acute post-exercise period. *Nutrition* 30: 961-967, 2014.
21. Gonzalez JT, Fuchs CJ, Smith FE, Thelwall PE, Taylor R, Stevenson EJ, Trenell MI, Cermak NM, and Van Loon LJ. Ingestion of glucose or sucrose prevents liver but not muscle glycogen depletion during prolonged endurance-type exercise in trained cyclists. *American Journal of Physiology-Endocrinology and Metabolism* 309: E1032-E1039, 2015.
22. Gonzalez JT, Richardson JD, Chowdhury EA, Koumanov F, Holman GD, Cooper S, Thompson D, Tsintzas K, and Betts JA. Molecular adaptations of adipose tissue to 6 weeks of morning fasting vs. daily breakfast consumption in lean and obese adults. *J Physiol* 596: 609-622, 2018.
23. Gonzalez JT, and Stevenson EJ. Assessment of the post-exercise glycemic response to food: considering prior nutritional status. *Nutrition* 2013.
24. Gonzalez JT, Veasey RC, Rumbold PL, and Stevenson EJ. Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. *British Journal of Nutrition* 110: 721-732, 2013.
25. Goodyear P, Laurie J, and Kahn M, Barbara B. Exercise, glucose transport, and insulin sensitivity. *Annual Review of Medicine* 49: 235-261, 1998.
26. Hamman L, and Hirschman I. Studies on blood sugar. *Archives of Internal Medicine* 20: 761-808, 1917.
27. Herzig S, and Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature reviews Molecular cell biology* 19: 121-135, 2018.
28. Holloszy JO. Exercise-induced increase in muscle insulin sensitivity. *Journal of applied physiology* 99: 338-343, 2005.
29. Impey SG, Hammond KM, Shepherd SO, Sharples AP, Stewart C, Limb M, Smith K, Philp A, Jeromson S, Hamilton DL, Close GL, and Morton JP. Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes. *Physiological Reports* 4: e12803, 2016.
30. Jentjens U, Achten, Currell, Mann, and Jeukendrup. Exogenous carbohydrate oxidation rates are elevated after combined ingestion of glucose and fructose during exercise in the heat. *Journal of Applied Physiology* 100: 807-816, 2006.
31. Jeukendrup A, and Wallis G. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *International Journal of Sports Medicine* 26: 28-37, 2005.
32. Jeukendrup AE, Wagenmakers AJM, Stegen JHCH, Gijsen AP, Brouns F, and Saris WHM. Carbohydrate ingestion can completely suppress endogenous glucose production during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 276: E672-E683, 1999.
33. Kahn CR, and White M. The insulin receptor and the molecular mechanism of insulin action. *The Journal of clinical investigation* 82: 1151-1156, 1988.
34. Knudsen SH, Karstoft K, Pedersen BK, van Hall G, and Solomon TP. The immediate effects of a single bout of aerobic exercise on oral glucose tolerance across the glucose tolerance continuum. *Physiological Reports* 2: Available online. doi: 10.14814/phy14812.12114, 2014.
35. Krusenstjerna-Hafstrom T, Madsen M, Vendelbo MH, Pedersen SB, Christiansen JS, Moller N, Jessen N, and Jorgensen JO. Insulin and GH signaling in

human skeletal muscle in vivo following exogenous GH exposure: impact of an oral glucose load. *PLoS One* 6: e19392, 2011.
36. Lee-Young RS, Palmer MJ, Linden KC, LePlastrier K, Canny BJ, Hargreaves M, Wadley GD, Kemp BE, and McConell GK. Carbohydrate ingestion does not alter skeletal muscle AMPK signaling during exercise in humans. *American Journal of Physiology-Endocrinology and Metabolism* 291: E566-E573, 2006.
37. Lee S-H, Tura A, Mari A, Ko S-H, Kwon H-S, Song K-H, Yoon K-H, Lee K-W, and Ahn Y-B. Potentiation of the early-phase insulin response by a prior meal contributes to the second-meal phenomenon in type 2 diabetes. *American Journal of Physiology-Endocrinology and Metabolism* 301: 984-990, 2011.
38. Liljeberg Elmstahl H, and Bjorck I. Milk as a supplement to mixed meals may elevate postprandial insulinaemia. *Eur J Clin Nutr* 55: 994-999, 2001.
39. Loftus GR, and Masson MEJ. Using confidence intervals in within-subject designs. *Psychonomic Bulletin & Review* 1: 476-490, 1994.
40. Mace OJ, Affleck J, Patel N, and Kellett GL. Sweet taste receptors in rat small intestine stimulate glucose absorption through apical GLUT2. *The Journal of Physiology* 582: 379-392, 2007.
41. Matsuda M, and DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22: 1462-1470, 1999.
42. McBride A, Ghilagaber S, Nikolaev A, and Hardie DG. The Glycogen-Binding Domain on the AMPK β Subunit Allows the Kinase to Act as a Glycogen Sensor. *Cell Metabolism* 9: 23-34, 2009.
43. Montain SJ, Hopper M, Coggan AR, and Coyle EF. Exercise metabolism at different time intervals after a meal. *Journal of Applied Physiology* 70: 882-888, 1991.
44. Moore MC, Smith MS, Farmer B, Coate KC, Kraft G, Shiota M, Williams PE, and Cherrington AD. Morning Hyperinsulinemia Primes the Liver for Glucose Uptake and Glycogen Storage Later in the Day. *Diabetes* db170979, 2018.
45. Moore MC, Smith MS, Farmer B, Kraft G, Shiota M, Williams PE, and Cherrington AD. Priming Effect of a Morning Meal on Hepatic Glucose Disposition Later in the Day. *Diabetes* 66: 1136-1145, 2017.
46. Nilsson M, Elmstahl H, and Bjorck I. Glucose and insulin responses to porridge and gruel meals intended for infants. *Eur J Clin Nutr* 59: 646-650, 2005.
47. Ning F, Zhang L, Dekker JM, Onat A, Stehouwer CD, Yudkin JS, Laatikainen T, Tuomilehto J, Pyörälä K, and Qiao Q. Development of coronary heart disease and ischemic stroke in relation to fasting and 2-hour plasma glucose levels in the normal range. *Cardiovascular Diabetology* 11: 76-84, 2012.
48. Pekkanen J, Tuomilehto J, Qiao Q, Jousilahti P, Lindström J, and Group DS. Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. *Lancet* 354: 617-621, 1999.
49. Ploug T, Van Deurs B, Ai H, Cushman SW, and Ralston E. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *The Journal of cell biology* 142: 1429-1446, 1998.
50. Radziuk J. An integral equation approach to measuring turnover in nonsteady compartmental and distributed systems. *Bulletin of Mathematical Biology* 38: 679-693, 1976.
51. Radziuk J, Norwich KH, and Vranic M. Experimental validation of measurements of glucose turnover in nonsteady state. *American Journal of Physiology-Endocrinology and Metabolism* 234: E84, 1978.
52. Richter EA, and Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological Reviews* 93: 993-1017, 2013.

53. Rizza RA, Toffolo G, and Cobelli C. Accurate Measurement of Postprandial Glucose Turnover: Why Is It Difficult and How Can It Be Done (Relatively) Simply? *Diabetes* 65: 1133-1145, 2016.
54. Rose AJ, Howlett K, King DS, and Hargreaves M. Effect of prior exercise on glucose metabolism in trained men. *American Journal of Physiology-Endocrinology and Metabolism* 281: 766-771, 2001.
55. Ruge T, Hodson L, Cheeseman J, Dennis AL, Fielding BA, Humphreys SM, Frayn KN, and Karpe F. Fasted to fed trafficking of fatty acids in human adipose tissue reveals a novel regulatory step for enhanced fat storage. *The Journal of Clinical Endocrinology & Metabolism* 94: 1781-1788, 2009.
56. Steele R, Bjerknes C, Rathgeb I, and Altszuler N. Glucose uptake and production during the oral glucose tolerance test. *Diabetes* 17: 415-421, 1968.
57. Tarnopolsky MA, Pearce E, Smith K, and Lach B. Suction-modified Bergström muscle biopsy technique: Experience with 13,500 procedures. *Muscle & nerve* 43: 716-725, 2011.
58. Treebak JT, Pehmøller C, Kristensen JM, Kjøbsted R, Birk JB, Schjerling P, Richter EA, Goodyear LJ, and Wojtaszewski JF. Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle. *The Journal of physiology* 592: 351-375, 2014.
59. Van Thienen R, D'Hulst G, Deldicque L, and Hespel P. Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiological Reports* 2: 2014.
60. Van Wijck K, Lenaerts K, Van Loon LJ, Peters WH, Buurman WA, and Dejong CH. Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. *PloS One* 6: doi 10.1371/journal.pone.0022366, 2011.
61. Vendelbo MH, Møller AB, Treebak JT, Gormsen LC, Goodyear LJ, Wojtaszewski JFP, Jørgensen JOL, Møller N, and Jessen N. Sustained AS160 and TBC1D1 phosphorylations in human skeletal muscle 30 min after a single bout of exercise. *Journal of Applied Physiology* 117: 289-296, 2014.
62. Wee S-L, Williams C, Tsintzas K, and Boobis L. Ingestion of a high-glycemic index meal increases muscle glycogen storage at rest but augments its utilization during subsequent exercise. *Journal of Applied Physiology* 99: 707-714, 2005.
63. Wolfe RR. Radioactive and stable isotope tracers in biomedicine: principles and practice of kinetic analysis. 1992.

CHAPTER 4 - BLOOD SAMPLING METHODS

Prior exercise alters the difference between arterialised and venous glycaemia - implications for blood sampling procedures.

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Acknowledgements:

The authors thank the volunteers for their time and effort in participating in this study. We also thank Iain Templeman, Oliver J. Perkin and Harriet A. Carroll for assistance with blood sampling during some trials.

Sources of Support:

This study was supported by The European Society for Clinical Nutrition and Metabolism and The Rank Prize Funds.

Trial Registration: https://clinicaltrials.gov/ [Number: NCT02852044].

Data Access Statement: The complete data set relating to this manuscript can be found at: https://researchdata.bath.ac.uk/352/

RELEVANCE TO THESIS

In physiology research, blood is frequently sampled to measure concentrations of metabolites and hormones to assess glucose tolerance or insulin sensitivity (215). However, studies aiming to answer broadly similar questions often use different methods to obtain blood samples (**Table 4.1**). Whilst arteries are a preferred sampling site for assessing the exposure of peripheral tissues to glucose and insulin, antecubital veins are a more common blood sampling site, largely due to risks associated with arterial cannulation, such as greater bleeding from the sampling site and blood clots (378). However, heating the hand to 37 ºC causes capillaries in the hand to vasodilate to disperse heat and this produces venous blood from the veins of the heated hand that has a gaseous composition that does not clearly differ from arterial blood (428). These are known as 'arterialised' or 'arterialised-venous' blood samples and provide concentrations of metabolites (e.g. glucose) and hormones (e.g. insulin) that do not clearly differ to concentrations in arterial samples that are collected simultaneously (380-382). Despite this, many research studies continue to measure glucose and insulin in non-arterialised venous blood (**Table 4.1**). This is a consideration if comparing research studies that have used different blood sampling methods.

For example, as outlined in **Chapter 1**, the ingestion of carbohydrates increases blood glucose concentrations and insulin is secreted to regulate this response, by suppressing glucose appearance from the liver and increasing blood glucose disposal into the peripheral tissues (215). Systemic glucose and insulin are transported in the blood to the periphery by arteries where insulin binds to the cell membranes and increases glucose disposal into peripheral tissues, whereas veins carry the blood back to the lungs after this interaction (194). Consequently, concentrations of glucose and insulin are higher when measured in arterialised *versus* venous blood (380-382) due to a partial removal of glucose by the peripheral tissues for storage or oxidation and insulin binding to cell membranes (194)**.** If differences between blood sample methods were the same across all testing conditions and populations, correction factors could be easily applied for comparisons of studies where different blood sampling methods have been used.

However, venous concentrations of metabolites and hormones can differ due to an uptake or release of metabolites and hormones from the tissue beds in close proximity to the sampling site. The magnitude of arterialised-venous differences also depends on the context in which a sample was collected. The determining factor is the interaction of tissues in proximity to the sampling site with the blood (**Figure 4.1**). As such, when comparing blood sampled after a meal or a hyperinsulinaemic-euglycaemic clamp, arterialised-venous differences for blood glucose concentrations are often greater than during fasting, due to an increased extraction of blood glucose by peripheral tissues (429). This may also vary across populations, due to differences in the capacity of muscle for disposing of glucose (430). Arterial-venous differences for concentrations of glucose may also depend on the activity status of participants as exercise increases net glucose uptake into active muscle (194) and can impair insulin sensitivity in non-active muscle (431).

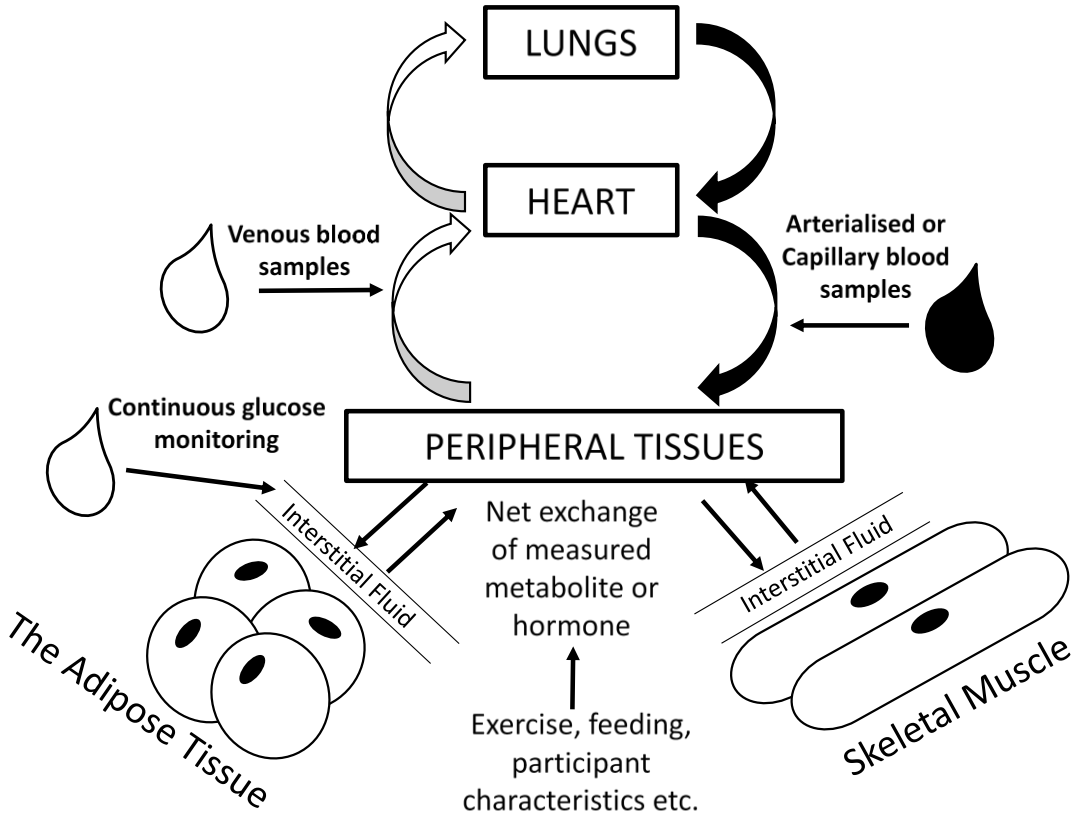

Figure 4.1. Different blood sampling methods produce different concentrations of metabolites and hormones due to the location of the sample site in relation to peripheral tissues. The magnitude of this difference between sampling methods depends on the characteristics of participants and their feeding and activity status and these will alter the interaction of the peripheral tissues with the blood (432).

Table 4.1. Studies that validated commonly-applied insulin sensitivity indices or have manipulated the activity/nutritional status of participants and assessed oral glucose tolerance or insulin sensitivity from fasting or postprandial blood samples.

Study details	Blood sampling method	Reported outcome
Stumvoll *et al.,* 2000	Venous (OGTT)	Stumvoll (validation)
Katz *et al.,* 2000	Venous (Fasting)	QUICKI (validation)
Matsuda & DeFronzo, 1999	Unclear (OGTT)	Matsuda (validation)
Belfoire *et al.,* 1998	Unclear (OGTT)	Belfoire (validation)
Cederholm & Wibell, 1990	Venous (OGTT)	Cederholm (validation)
Roberts *et al.,* 2016	Arterialised (OGTT)	Glucose AUC
Wright *et al.,* 2016	Unclear (OGTT)	Matsuda & HOMA-IR
Martin *et al.,* 2016	Venous (OGTT)	Glucose AUC
Shephard *et al.,* 2016	Venous (OGTT)	Glucose AUC
Campbell *et al.,* 2015	Interstitial Fluid (Free Living)	Glucose AUC
Khattab *et al.,* 2015	Unclear (OGTT)	Glucose AUC
Cermak *et al.,* 2015	Venous (OGTT)	Matsuda
Gonzalez *et al.,* 2015	Capillary (OGTT)	Matsuda & HOMA2-IR
Ross *et al.,* 2015	Unclear (OGTT)	Matsuda
Gonzalez & Stevenson, 2014	Arterialised (Mixed Meal)	Glucose AUC
Knudsen *et al.,* 2014	Venous (OGTT)	Glucose AUC
Ortega *et al.,* 2014	Venous (OGTT)	Matsuda
Mottola *et al.,* 2013	Venous (OGTT)	Matsuda
Van Dijk *et al.,* 2013	Venous (Mixed Meal)	Glucose AUC
Numao *et al.,* 2013	Venous (OGTT)	Matsuda & Stumvoll
Gonzalez *et al.,* 2013	Venous (Mixed Meal)	Matsuda
Walhin *et al.,* 2013	Venous (OGTT)	Matsuda
Weinheimer *et al.,* 2012	Unclear (OGTT)	Matsuda & HOMA-IR
Dunstan *et al.,* 2012	Venous (Mixed Meal)	Glucose AUC

Investigating whether prior exercise alters the magnitude of arterialised-venous differences for blood glucose was important in this thesis. Consuming breakfast before exercise was previously shown to increase blood glucose concentrations at a post-exercise meal, compared to when breakfast was consumed at rest (15) (**Figure 4.2A**). However, this blood glucose response to a post-exercise meal with exercise *versus* rest was not shown in **Chapter 3** (**Figure 4.2B**), despite both studies recruiting young, physically active men and prescribing a similar duration and intensity of exercise (1 h, moderate-intensity) in the same proximity to the post-exercise meal (1 h prior). The exercise mode might explain this result, as running was used in the first study, and cycling was used in **Chapter 3** and this may have altered the magnitude of the intestinal damage response to exercise and consequently the blood glucose rate of appearance (292). In **Chapter 3**,

lower postprandial intestinal fatty acid binding protein concentrations in blood were measured with breakfast consumption before exercise, suggesting a lower intestinal damage response compared to breakfast omission before exercise. If running induces a different intestinal damage response to cycling and with prior breakfast consumption *versus* omission needs investigation. However, another difference between the studies was the sampling method used to obtain blood, with venous blood in previous work (15) and arterialised-venous blood sampled in **Chapter 3**, evident by the higher measured systemic glucose concentrations in **Figure 4.2**. If exercise alters the net uptake of blood glucose by the tissues in proximity to the sampling site, this may explain why a heightened postprandial blood glucose response to a post-exercise meal compared to rest was apparent in previous work with venous blood (so after tissue-blood interactions) but not in **Chapter 3** with arterialised blood (so before tissue-blood interactions). However, a study of arterialised-venous differences for blood glucose concentrations after exercise *versus* rest had not been completed, so was the aim of **Chapter 4**.

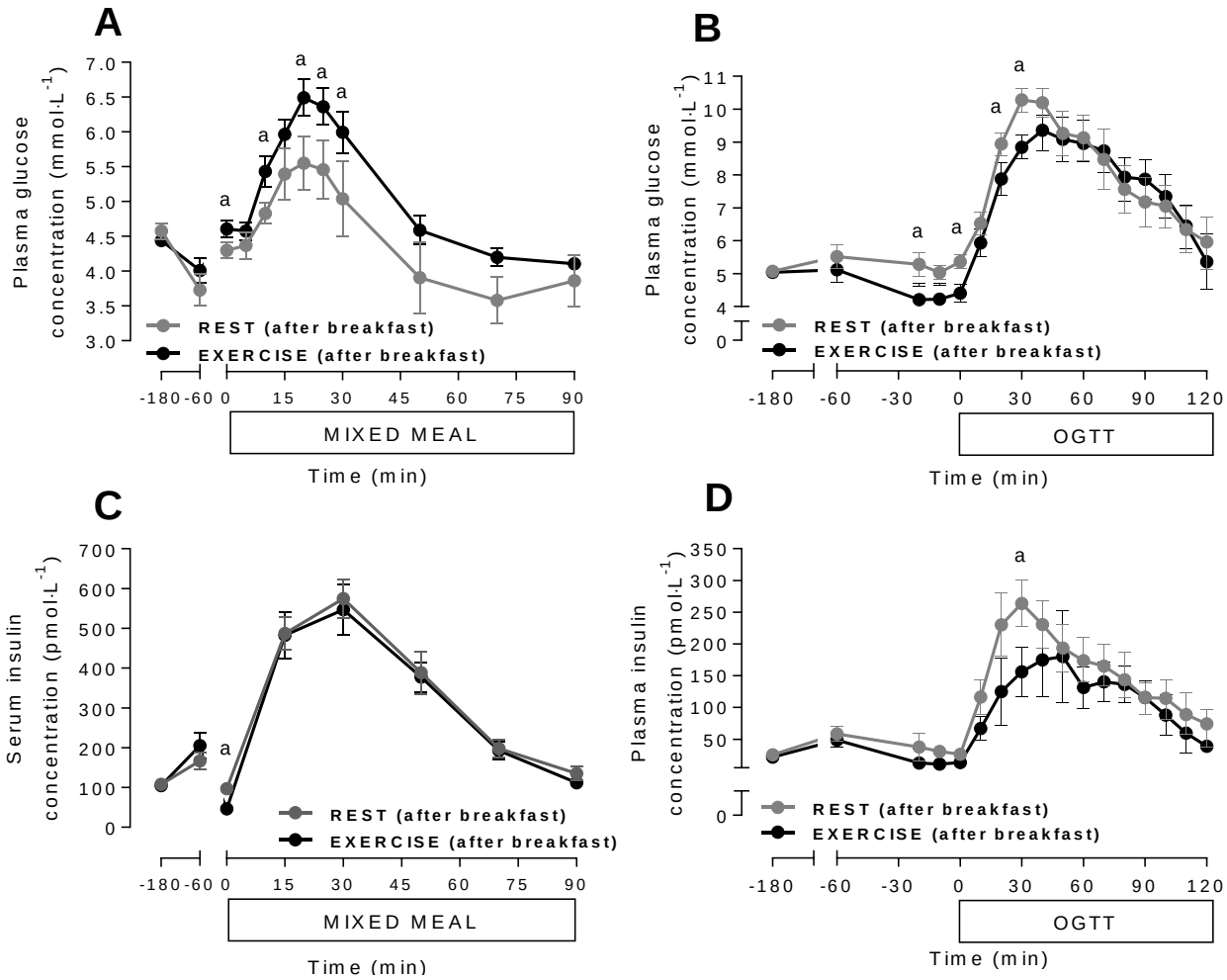

Figure 4.2. Postprandial blood glucose (**A and B**) and insulin (**C and D**) concentrations after rest and exercise with prior breakfast consumption. In panels **A and C**, data are means ± 95% confidence intervals (CI) for *n*=11 healthy men and are redrawn from Gonzalez *et al.* (15). In panels **B and D**, data are redrawn from **Chapter 3** and are means ± normalised 95% CI for *n*=12 healthy men. 'a' represents a difference between rest and exercise with $p<0.05$.

This declaration concerns the article entitled:									
Prior exercise alters the difference between arterialised and venous glycaemia - implications for blood sampling procedures.									
Publication status (tick one)									
Draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	**X**
Publication details (reference)	Edinburgh, R. M., Hengist, A., Smith, H. A., Betts, J. A., Thompson, D., Walhin, J. P., & Gonzalez, J. T. (2017). Prior exercise alters the difference between arterialised and venous glycaemia: Implications for blood sampling procedures. *British Journal of Nutrition*, *117(10)*, 1414-1421.								
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Candidate's contribution to the paper (provide details, and also indicate as a percentage)	**Formulation of ideas:** RME and JTG formulated the ideas for the study **Design of methodology:** RME and JTG predominantly designed the methodology. **Experimental work:** RME (> 95 %), JTG, AH, HS and J-PW conducted the research, RME and JTG analyzed the data, RME was present for all data collection on trial days, prepared and analysed the blood samples (for metabolites and hormones), completed data and statistical analysis and was heavily involved in all aspects of the experimental work. **Presentation of data in journal format:** RME (> 80 %) and JTG wrote the paper, and all authors contributed to earlier versions of the manuscript and approved the final version of the manuscript.								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
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4172

ABSTRACT

Oral glucose tolerance and insulin sensitivity are common measures, but are determined using various blood sampling methods, employed under many different experimental conditions. This study established whether measures of oral glucose tolerance and oral glucose-derived insulin sensitivity (ISI) differ when calculated from venous *versus* arterialised blood. Critically, we also established whether any differences between sampling methods are consistent across distinct metabolic conditions (after rest *versus* after exercise). Ten healthy men completed two trials in a randomised order, each consisting of a 120-min oral glucose tolerance test (OGTT), either at rest or post-exercise. Blood was sampled simultaneously from a heated hand (arterialised) and an antecubital vein of the contralateral arm (venous). Under both conditions, glucose time-averaged area under the curve was greater from arterialised compared to venous plasma but importantly, this difference was larger after rest relative to after exercise (0.99 ± 0.46 *versus* 0.56 ± 0.24 mmol·L^{-1} respectively; $p < 0.01$). OGTT-derived $ISI_{Matsuda}$ and $ISI_{Cederholm}$ were lower when calculated from arterialised relative to venous plasma and the arterialised-venous difference was greater after rest *versus* after exercise ($ISI_{Matsuda}$: 1.97 ± 0.81 *versus* 1.35 ± 0.57 au, respectively; $ISI_{Cederholm}$: 14.76 ± 7.83 *versus* 8.70 ± 3.95 au, respectively; both $p < 0.01$). Venous blood provides lower postprandial glucose concentrations and higher estimates of insulin sensitivity, compared to arterialised blood. Most importantly, these differences between blood sampling methods are not consistent after rest *versus* post-exercise, preventing standardised venous-to-arterialised corrections from being readily applied.

INTRODUCTION

Insulin resistance is the first detectable defect in the pathogenesis of metabolic diseases [e.g. type 2 diabetes (T2D) (1)] and can, along with the associated impairment in glucose tolerance, independently predict risk of cardiovascular disease and mortality (2). Glucose tolerance and insulin sensitivity can be quantified *in vivo* using an oral glucose tolerance test (OGTT). Indeed, the OGTT is frequently employed in both clinical (to diagnose T2D) and research settings (to assess the efficacy and/or effectiveness of exercise or dietary interventions for altering blood glucose control). After an overnight fast, a 75 g glucose load is ingested and followed by an observation period (usually 120 min) where blood samples are collected to determine glucose concentrations. If combined with insulin measurements, estimates of whole-body insulin sensitivity can be derived that are more representative of free-living physiology than other measures such as the hyperinsulinemic-euglycemic clamp (3-6).

However, guidelines for the OGTT provide no clear consensus or justification of the most appropriate method for blood sampling. Consequently, oral glucose tolerance and OGTT-derived estimates of insulin sensitivity are determined from blood sampled from antecubital veins (7), capillaries (8), heated dorsal hand veins (9), and glucose concentrations in the interstitial fluid (9). In some instances, the blood sampling method (i.e. the sample site used) is not reported, including in the validation of a commonly cited (> 2500 times) oral glucose derived insulin sensitivity index (5). Arterial blood best represents the exposure of peripheral tissues such as skeletal muscle to systemic metabolites and hormones. When knowledge of peripheral exposure to glucose and insulin is required, arterial samples may therefore be preferable. However, due to the potential risks associated with arterial cannulation (10), alternative methods are often used to provide blood reflective of arterial samples (commonly referred to as arterialised-venous or arterialised blood), including the heated-hand technique (11-17). Nonetheless, many studies derive oral glucose tolerance and insulin sensitivity from non-arterialised venous blood from an antecubital vein, under conditions where the activity and/or feeding status of participants is manipulated

(7). Some guidelines provide corrections for venous to capillary plasma for an OGTT, but only at rest (18). However, concentrations of metabolites or hormones in a vein can differ due to their net uptake or release from tissue beds, which depends on environment, nutritional and/or metabolic factors. For example, a bout of endurance-type exercise increases insulin sensitivity and glucose uptake in active muscle (19) and can impair insulin sensitivity in non-exercised muscle (20). This prohibits venous-to-arterialised corrections, or direct comparisons between studies that have used different, but common, methods for obtaining blood samples (i.e. arterialised blood from a heated hand vein *versus* blood from an antecubital vein).

A comparison of arterialised and venous blood, for estimates of glucose tolerance and OGTT-derived insulin sensitivity, under different metabolic conditions (i.e. when prior exercise has been completed) has never been performed. As such, the aim of this study was to: 1) establish if OGTT-derived insulin sensitivity indices differ when calculated from arterialised *versus* venous blood samples and 2) investigate whether prior lower-limb exercise influences the magnitude of any differences between arterialised and venous concentrations of glucose or insulin.

METHODS

Trial Design

This study adopted a cross-over design, whereby participants completed preliminary measures followed by exercise and rest trials in a randomised order (randomisation performed by RME using an online tool: randomizer.org), separated by an interval of 7-21 days. On main trial days, an hour of moderate-intensity cycling or rest (lying recumbent on a bed) was followed by an oral glucose tolerance test (OGTT), during which blood samples were collected simultaneously from a pre-heated dorsal hand vein (arterialised) and an antecubital vein of the contralateral arm (venous). All trials were completed at the University of Bath (Bath, UK) in accordance with the Declaration of Helsinki. The study was approved by the Research Ethics Approval Committee for Health at the University of Bath (reference: EP 15/16 44).

Participants

Ten self-reported physically active and healthy men (mean ± SD: age: 23 ± 3 years; body mass: 76.9 ± 5.7 kg; height: 181.6 ± 4.5 cm; body mass index: 23.3 ± 1.8 $kg \cdot m^{-2}$; $\dot{V}O_2$ peak: 52.7 ± 8.9 $ml \cdot kg^{-1} \cdot min^{-1}$) were recruited from the staff and student population at the University of Bath, between November 2015 and April 2016. Exclusion criteria included any history of metabolic disease, or any condition that might have posed undue personal risk to the participant or introduced bias to the experiment. Written, informed consent was obtained from all participants prior to testing.

Preliminary Testing

Upon arrival at the laboratory, height was measured to the nearest 0.1 cm with a stadiometer (Seca Ltd, Birmingham, UK), with participants barefoot in the Frankfurt plane. Body mass was measured with participants in light clothing and to the nearest 0.1 kg using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). An incremental cycling test was then completed at a self-selected cadence on an electronically-braked ergometer (Excalibur Sport, Lode®, Groningen, Netherlands). Participants were allowed to adjust the saddle and handlebar heights to their preferred position. The initial power output was set at 50 W and was increased by 50 W every four min, for four stages. Thereafter, the power output was increased by 20 W every min until volitional exhaustion. Heart rate was monitored throughout (Polar Electro Oy, Kempele, Finland) and breath-by-breath measurements were recorded using an online gas analysis system (TrueOne2400, ParvoMedics, Sandy, USA). The volume and gas analysers used were calibrated with a 3 L calibration syringe (Hans Rudolph, Kansas City, USA) and known concentrations of a calibration gas (16.04 % O_2; 5.06 % CO_2) respectively. Maximal power output (MPO) was taken as the work rate of the last completed stage, plus the fraction of time in the final non-completed stage, multiplied by the work rate increment. Peak oxygen uptake ($\dot{V}O_2$ peak) was taken as the highest average over a rolling 30 second period.

Main Trials

Participants arrived at 0800 ± 1 hour, after a minimum 10 hour overnight fast and having refrained from strenuous physical activity and caffeine for 24 hours. They were asked to record their evening meal and physical activity on the day before the first trial and replicate this for the second visit, in line with standard procedures for postprandial glucose tolerance testing (21). Upon arrival, participants placed their dominant hand into a heated-air box set to a constant temperature of 55°C (Mass Spectrometry Facility; The University of Vermont & the University of Vermont Medical Center, Burlington, USA). After 20 minutes, intravenous cannulae (BD Venflon Pro, BD, Helsingborg, Sweden) were fitted in the heated dorsal hand vein (retrograde) and the antecubital fossa of the contralateral arm (antegrade). A simultaneous baseline 5 mL blood sample was drawn from each site to be used as a baseline sample. Cannulae were kept patent via flushing of a 0.9 % sodium chloride infusion (B.Braun, Melsungen, Germany) and the first 2 mL of each sample was thus discarded. Plasma was obtained by dispensing whole blood into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) which were centrifuged for 10 minutes (4°C and 3500 *g*) (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Samples were dispensed into 0.5 mL aliquots and immediately frozen at -20°C, before longer-term storage at -80°C. On the exercise trial, participants completed 60 minutes of cycling on an ergometer (Monark 894 E, Monark, Vansbro, Sweden) at 50 % MPO. On the rest trial, they remained in the recumbent position throughout this period and were permitted to complete resting activities (i.e. watching television or reading).

In both trials, an OGTT was then performed. Participants ingested 82 g of dextrose powder (Myprotein, Northwich, UK) dissolved in 300 mL of water (equivalent to 75 g of anhydrous glucose). Whole blood (5 mL) was collected immediately prior to (OGTT 0 minute) and every 15 minutes post-ingestion of the glucose load from the heated dorsal hand vein and the antecubital vein of the contralateral arm simultaneously, and processed via aforementioned methodology. Expired gas samples were collected at baseline and hourly during the OGTT for five minutes, and for one minute at 15 minute intervals during

exercise (and same time points in the rest trial). Gas samples were collected into 200 L Douglas Bags (Hans Rudolph, Kansas City, USA) via falconia tubing (Baxter, Woodhouse and Taylor Ltd, Macclesfield, UK). Concurrent measurements of inspired air composition were made, to correct for changes in ambient O_2 and CO_2 concentrations. Expired O_2 and CO_2 concentrations were measured in a known volume of each sample via paramagnetic and infrared analysers (Mini HF 5200, Servomex Ltd., UK).

Data Analysis

Plasma glucose, lactate and triglyceride concentrations were measured on an automated analyser (Daytona; Randox Lab, Crumlin, UK) according to manufacturer's instructions. Plasma insulin concentrations were determined using a commercially available ELISA (Mercodia AB, Uppsala, Sweden; intra-assay CV, 3.7 %; inter-assay CV 6.5 %). Samples were analysed in batch after all data collection was completed, and for every participant, all samples from both trials were analysed on the same plate. Energy expenditure and substrate utilisation were determined for rest, exercise and during the OGTT via indirect calorimetry. Assuming urinary nitrogen excretion to be negligible, substrate utilisation was calculated using stoichiometric equations and adjusted for exercise values to account for the contribution of glycogen (22). At rest, these calculations assume that glucose provides all of the carbohydrate required for metabolism. Adjustments were then made for estimations of carbohydrate oxidation during moderate-intensity exercise, where metabolic requirements are met by glucose and glycogen, which typically provide a 20 and 80% contribution respectively (22). Energy expenditure was determined assuming that lipids, glucose and glycogen provide 9.42, 3.74 and 4.15 $kcal \cdot g^{-1}$ (22).

Statistical Analysis

A sample size estimation was performed using fasting glucose concentrations sampled from arterialised and venous blood (mean ± SD: 4.70 ± 0.19 *versus* 4.52 ± 0.19 $mmol \cdot L^{-1}$, for arterialised *versus* venous, respectively) (13). Using the calculated effect size of 1.33, ten participants were required to provide an 80 %

chance of detecting a statistical difference between arterialised and venous blood for glucose with an α-level of 0.05. The area underneath the concentration-time curve (AUC) for the plasma glucose and plasma insulin OGTT responses was calculated using the trapezoid rule. The total AUC (mmol·120min·L^{-1}) was divided by the duration of the OGTT observation period (120 min) to provide a time-averaged AUC (mmol·L^{-1}), which was used as a summary measure for the reported postprandial responses. Insulin sensitivity indices were calculated as described in the relevant table or figure (4-6, 23, 24). For comparisons of multiple means, a two-way repeated measures trial (exercise or rest) x sample method (arterialised or venous) ANOVA was employed. Where significant trial x sample method interactions were detected, *t*-tests were performed to locate variance, and corrected for multiple comparisons using Sidak's multiple comparison test. A Pearson *r* was used to correlate the arterialised-venous difference after rest against the arterialised-venous difference after exercise. Data are presented as means ± SD in text and means ± 95 % confidence intervals in figures unless otherwise stated. All statistical analysis was completed using a commercially available software package (GraphPad Software, Inc, CA, USA). In one participant's exercise trial the 105 minute and 120 minute post OGTT blood samples were not collected from both sampling methods, and for these time points (1 % of samples collected across the study) the last observation carried forward approach was used. Statistical significance was accepted at $p \leq 0.05$.

RESULTS

Energy expenditure and substrate utilisation

The exercise was completed as prescribed, except for one participant for whom the intensity was decreased to 45 % MPO for the final 30 minutes due to his inability to maintain an intensity of 50 % MPO. The exercise intensity was equivalent to 63 ± 5 % $\dot{V}O_2$ peak. In the rest trial, energy expenditure was 5.68 ± 0.55 kJ·min^{-1} in the hour before the OGTT and rates of carbohydrate and fat oxidation were 0.14 ± 0.07 and 0.09 ± 0.04 g·min^{-1}, respectively. In the exercise trial, energy expenditure during cycling was 51.00 ± 5.51 kJ·min^{-1} and carbohydrate and fat oxidation rates were 2.21 ± 0.41 and 0.38 ± 0.16 g·min^{-1} respectively. During the OGTT, no difference was detected between trials in energy expenditure or substrate oxidation (all $p > 0.05$).

Plasma glucose concentrations

At baseline, there was no difference in plasma glucose concentrations measured in arterialised compared to the venous samples in the rest (5.31 ± 0.35 *versus* 5.27 ± 0.28 mmol·L^{-1} respectively, $p > 0.05$) or exercise trials (5.25 ± 0.47 *versus* 5.18 ± 0.37 mmol·L^{-1} respectively, $p > 0.05$). Immediately prior to the OGTT, plasma glucose concentrations were higher in arterialised compared to venous samples after rest (**Table 1**; $p = 0.02$) but not after exercise ($p > 0.05$). After rest, glucose concentrations were higher at 30 and 45 minutes post-OGTT in arterialised relative to venous plasma (**Figure 1A**; all $p < 0.05$). In the exercise trial glucose concentrations did not differ between arterialised or venous plasma at any time post-glucose ingestion (**Figure 1B**; all $p > 0.05$). Glucose AUC was higher when calculated from arterialised relative to venous plasma after rest and after exercise (**Figure 3A**; both $p < 0.01$). However, the arterialised-venous difference was greater after rest (0.99 ± 0.46 mmol·L^{-1}) relative to after exercise (0.56 ± 0.24 mmol·L^{-1}) (**Figure 3A**; $p < 0.01$). When analysed independently, 120 minute post-OGTT glucose concentrations were higher in arterialised relative to venous plasma, and in both trials (Table 2; $p < 0.05$). When recommended adjustments for venous to capillary plasma were applied [venous plasma + 1.1 mmol·L^{-1} (18)], glucose concentrations were higher in venous *versus* arterialised

plasma (**Table 1**; $p < 0.05$). For the plasma glucose AUC, the magnitude of the arterialised-venous difference after rest was correlated with the magnitude of the arterialised-venous difference after exercise ($r = 0.800$, $p < 0.01$).

Plasma insulin concentrations

At baseline, there was no difference in plasma insulin concentrations measured in arterialised relative to the venous samples in the rest (21.26 ± 4.26 *versus* 20.49 ± 4.47 $pmol \cdot L^{-1}$ respectively, $p > 0.05$) or exercise trials (22.39 ± 6.78 *versus* 21.55 ± 5.93 $pmol \cdot L^{-1}$ respectively, $p > 0.05$). Thereafter, there were no difference in plasma insulin concentrations measured in arterialised compared to venous samples at any time point in the rest (**Figure 2A**) or exercise trials (**Figure 2B**; both $p > 0.05$). The time-averaged insulin AUC was higher in arterialised plasma relative to venous plasma after rest and after exercise (**Figure 3B**; both $p < 0.01$). No trial x sample method interaction was detected, indicating that the arterialised-venous difference was similar across the two trials (17.7 ± 16.8 *versus* 11.2 ± 6.7 $pmol \cdot L^{-1}$ for rest *versus* exercise, $p = 0.13$). For the plasma insulin AUC, the arterialised-venous difference after rest was correlated with the arterialised-venous difference after exercise ($r = 0.790$, $p < 0.01$). When data from both trials were pooled, the arterialised-venous difference for insulin AUC was correlated with the arterialised-venous difference in glucose AUC ($r = 0.766$, $p < 0.01$).

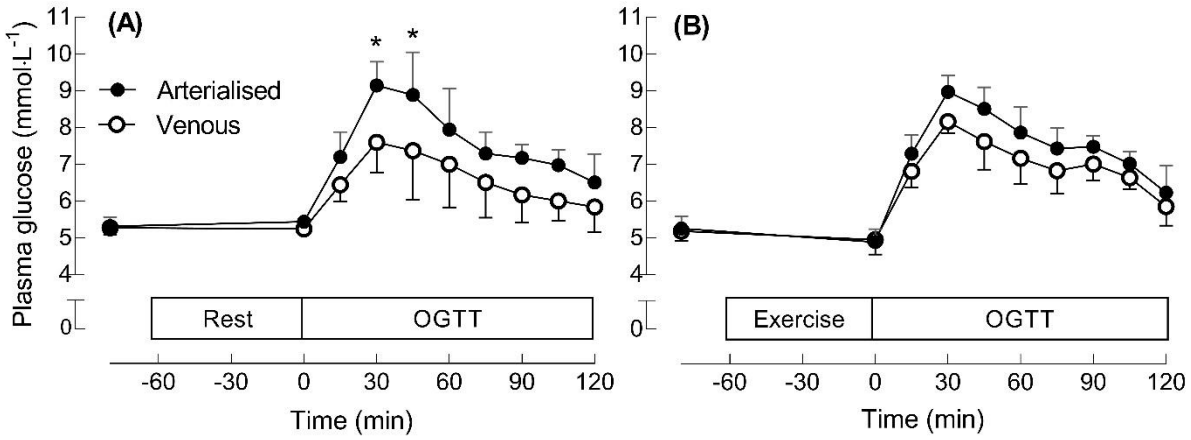

Figure 1. Plasma glucose concentrations during an oral glucose tolerance test (OGTT) after rest (**A**) or after exercise (**B**). Data are samples collected simultaneously from a pre-heated dorsal hand vein (arterialised) and the antecubital fossa of a contralateral arm (venous). Values are mean ± 95% confidence intervals. * Denotes significant differences between arterialised and venous samples with $p < 0.05$. $n = 10$.

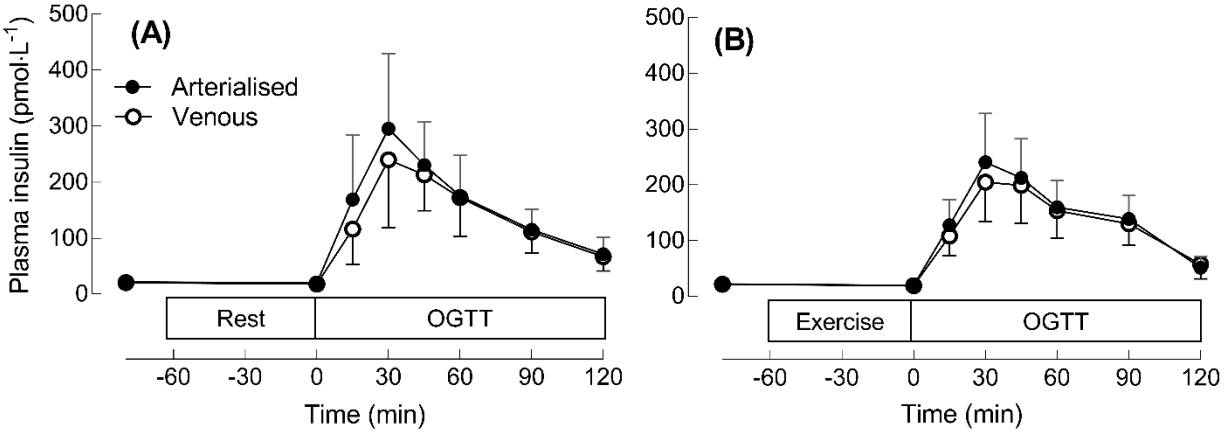

Figure 2. Plasma insulin concentrations during an oral glucose tolerance test (OGTT) after rest (**A**) or after exercise (**B**). Data are samples collected simultaneously from a pre-heated dorsal hand vein (arterialised) and the antecubital fossa of a contralateral arm (venous). Values are mean ± 95% confidence intervals. $n = 10$.

Table 1. Plasma concentrations and time-averaged area under concentration time curve for metabolites and insulin sensitivity indices for an oral glucose tolerance test.

	Rest		Exercise	
	Arterialised	***Venous***	***Arterialised***	***Venous***
OGTT 0 glucose (mmol·L^{-1})	5.43 (0.24)	5.24 (0.26) *	4.88 (0.47)	4.95 (0.53) †
Peak glucose (mmol·L^{-1})	9.55 (0.90)	8.17 (0.89) **	8.98 (0.62)	8.30 (0.47) ** †
120 min glucose (mmol·L^{-1})	6.50 (1.08)	5.84 (0.96) **	6.23 (1.04)	5.85 (0.73) *
Correct glucose (mmol·L^{-1})	6.50 (1.08)	6.94 (0.96) **	6.23 (1.04)	6.95 (0.73) **
OGTT 0 insulin (pmol·L^{-1})	20 (6)	18 (5) *	20 (5)	19 (6)
Peak insulin (pmol·L^{-1})	319 (179)	290 (152) **	247 (121)	223 (97) **
120 min insulin (pmol·L^{-1})	71 (42)	67 (36)	51 (28)	58 (37) †
OGTT 0 Lactate (mmol·L^{-1})	0.67 (0.18)	0.68 (0.17)	1.43 (0.74)	1.39 (0.59)
Lactate AUC (mmol·L^{-1})	0.95 (0.24)	0.99 (0.22)	1.09 (0.28)	1.11 (0.25)
OGTT 0 TAG (mmol·L^{-1})	0.59 (0.16)	0.62 (0.15)	0.67 (0.16)	0.72 (0.13) **
TAG AUC (mmol·L^{-1})	0.56 (0.18)	0.57 (0.17)	0.63 (0.17)	0.64 (0.17)
ISI Stumvoll (au)	0.12 (0.01)	0.13 (0.01) **	0.12 (0.01)	0.12 (0.01)
HOMA2-IR (au)	0.39 (0.12)	0.34 (0.11) *	0.37 (0.11)	0.36 (0.12)
QUICKI-IR (au)	0.41 (0.02)	0.42 (0.03) *	0.42 (0.03)	0.43 (0.04)

Data are samples collected simultaneously from a pre-heated dorsal hand vein (arterialised) and the antecubital fossa of the contralateral arm (venous). Values are mean (standard deviation). n = 10. TAG = Triglyceride.* equals a significant difference between arterialised and venous blood samples with $p < 0.05$. ** equals a significant difference between arterialised and venous blood samples with $p < 0.01$. † equals a significant sample method *versus* trial interaction with $p < 0.05$. $ISI_{Stumvoll}$ = 0.226 - [0.0032 x BMI (kg·m^{-2})] - [0.000064 x plasma insulin at OGTT 120 (mIU·ml^{1})] - [0.0037 x plasma glucose at OGTT 90 (mmol·L^{-1})]. QUICKI-IR = 1/ [log plasma insulin at OGTT 0 (mIU·ml^{-1}) + log plasma glucose at OGTT 0 (mg·dl^{-1})]. HOMA2-IR = calculated as per: https://www.dtu.ox.ac.uk/homacalculator/download.php

Insulin sensitivity indices

The OGTT-derived $ISI_{Matsuda}$ was lower if calculated from arterialised compared to venous plasma in both trials (**Figure 3C**; $p < 0.01$). A trial x sample method interaction indicated that the arterialised-venous difference was greater after rest (1.97 ± 0.81 au) compared to after exercise (1.35 ± 0.57 au) trial (**Figure 3C**; p = 0.03). Similarly, the $ISI_{Cederholm}$ was lower if calculated from arterialised relative to

venous plasma after rest and after exercise (**Figure 3D**; both $p < 0.01$). The arterialised-venous difference was also greater after rest compared to after exercise (14.76 ± 7.83 au *versus* 8.70 ± 3.95 au; **Figure 3D**; $p < 0.01$). There was a small but statistically significant difference for $ISI_{Strumvoll}$ after rest, with calculated values lower when derived from arterialised, relative to venous plasma ($p < 0.01$). After exercise, a similar trend was observed, but the difference between sampling methods did not reach statistical significance in this instance, and no significant trial x sample method interaction effect was detected for this outcome (**Table 1**; both $p = 0.08$). The HOMA2-IR index was higher if calculated from arterialised relative to venous plasma at rest (**Table 1**; $p = 0.02$) but no difference was detected between sample methods after exercise ($p > 0.05$). The QUICKI-IR index was lower if calculated from arterialised, relative to venous plasma after rest (**Table 1**; $p = 0.01$). A similar trend was also apparent after exercise, but the difference between sampling methods did not reach statistical significance in this instance ($p = 0.12$). No significant trial x sample method interaction effect was detected for HOMA2-IR or QUICKI-IR ($p > 0.05$).

Other Metabolites

Immediately prior to the OGTT, plasma lactate concentrations were not different in arterialised relative to venous samples, and this was apparent in both the exercise and rest trials (**Table 1**; both $p > 0.05$). Lactate AUC was not significantly different if calculated from arterialised or venous plasma after rest or after exercise (**Table 1**; both $p > 0.05$). Prior to the OGTT, plasma triglyceride concentrations were not affected by the sample method used after rest (**Table 1**; $p > 0.05$), but after exercise concentrations were higher in venous relative to arterialised samples ($p < 0.01$). Triglyceride AUC was unaffected by sample method after rest and exercise (**Table 1**; both $p > 0.05$). No trial x sample method interaction was detected for either the lactate or triglyceride AUC.

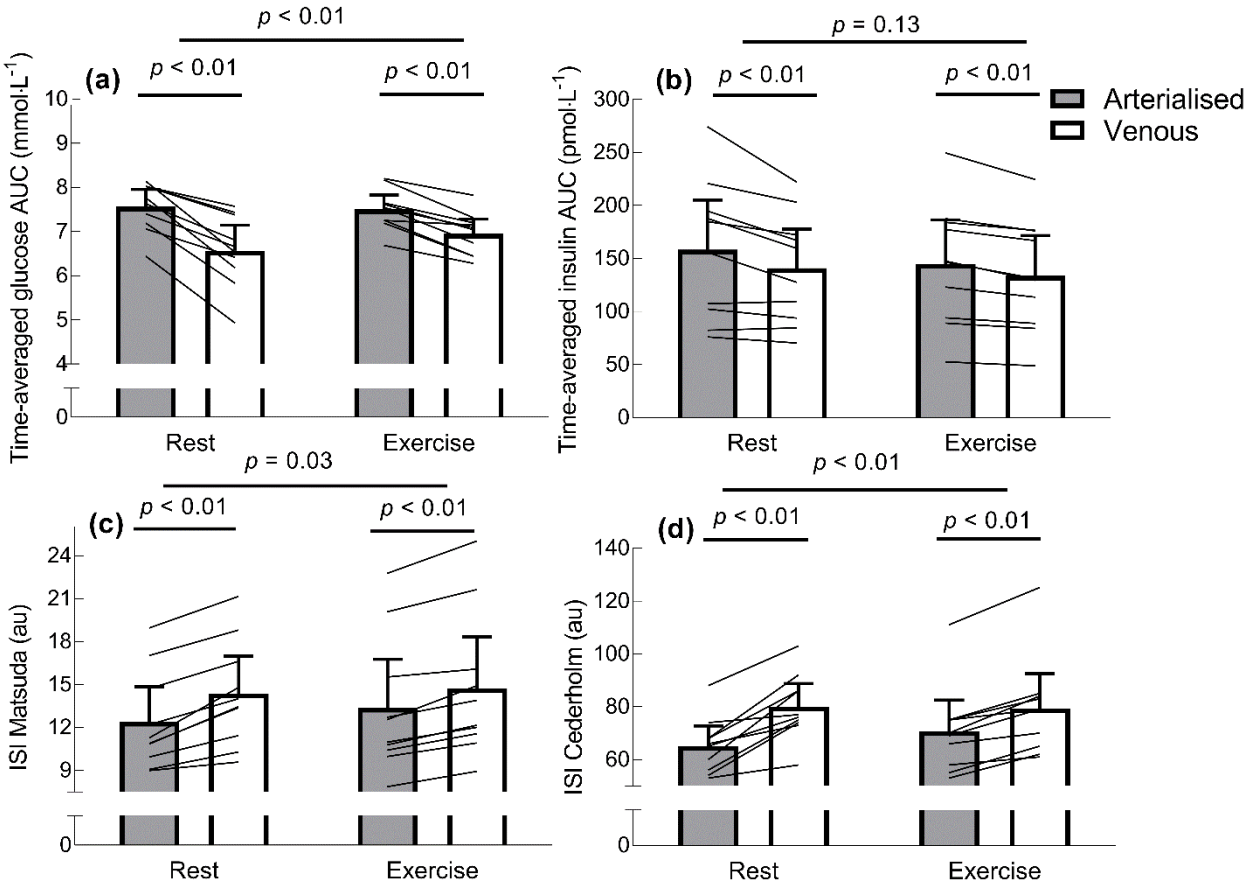

Figure 3. Time averaged area under the plasma glucose (**A**) and insulin (**B**) concentration time curves, and ISI Matsuda (**C**) and ISI Cederholm (**D**) indices after rest or exercise. Data are samples collected simultaneously from a pre-heated dorsal hand vein (arterialised) and the antecubital fossa of a contralateral arm (venous). Values are mean ± 95% confidence intervals. *n* = 10. $ISI_{Matsuda}$ = 10,000 / [baseline glucose ($mg \cdot dL^{-1}$) x baseline insulin ($mIU \cdot mL^{-1}$)] x [mean glucose over 120 minutes ($mg \cdot dL^{-1}$) x mean insulin over 120 minutes ($mIU \cdot mL^{-1}$)]. $ISI_{Cederholm}$ = 75000 + [baseline glucose ($mmol \cdot L^{-1}$) - glucose at OGTT 120 ($mmol \cdot L^{-1}$)] x 0.19 x 180 x 1.15 x Body Mass (kg) / [120 x log mean insulin over 120 minutes ($mIU \cdot mL^{-1}$) x mean glucose over 120 minutes ($mmol \cdot L^{-1}$)].

DISCUSSION

This study demonstrates that venous blood samples produce different values with respect to both glucose tolerance and insulin sensitivity indices, compared to arterialised blood samples. Furthermore, if assessing whole-body glucose tolerance, differences between sampling methods are not consistent under different metabolic conditions (e.g. when a prior bout of endurance-type exercise is performed, compared to rest) and so no single venous-to-arterialised correction factor can always be applied.

It has been known for decades that post-meal blood glucose concentrations can predict future risk of metabolic diseases (25). Thus, glucose tolerance and OGTT-derived insulin sensitivity indices are widely used in research to assess disease risk and responses to lifestyle or pharmacological interventions. Whilst arteries are the preferred site for determining peripheral exposure to metabolites or hormones, antecubital veins are a common site for postprandial blood sampling, due to risks associated with arterial cannulation. Nonetheless, it has been known since at least the 1920's that heating the hand to approximately 37ºC causes the cutaneous capillary beds to vasodilate as part of a homeostatic mechanism to radiate heat (26). This results in the dorsal veins of a heated hand providing concentrations of metabolites (e.g. glucose, non-esterified fatty acids, amino acids and lactate) and hormones (e.g. insulin and glucagon) that are consistent with arterial blood (11-17). Even increasing ambient temperature produces higher postprandial concentrations of glucose and insulin if sampled in venous blood, due to partial arterialisation (27, 28). The data presented here also show that postprandial glucose concentrations are higher if measured in arterialised, relative to venous plasma, but importantly the magnitude of this difference is influenced by the activity status of participants at the time when the OGTT is performed. This means that when glucose tolerance or insulin sensitivity are assessed with an OGTT, it is essential to report the blood sampling procedure that is used, as this could influence the interpretation of reported findings and will allow for more appropriate comparisons between studies that have used different blood sampling methods. Immediately prior to the OGTT, plasma glucose

concentrations were higher in arterialised relative to venous plasma after rest, but there was no difference between sample methods when a prior bout of exercise was performed. After rest, differences were detected when indices of insulin resistance that use fasting concentrations of glucose and insulin were calculated from arterialised *versus* venous plasma (i.e. HOMA2-IR and QUICKI), but the absence of a trial x sample method interaction suggests that the arterialised-venous difference is uninfluenced by activity status. In line with previous results, we showed that in both trials the postprandial glucose AUC was greater if calculated from arterialised, relative to venous plasma. Importantly, these novel data demonstrate that the arterialised-venous difference was lower if the OGTT was performed after a bout of exercise, compared to after rest. Therefore, when assessing glucose tolerance, venous samples cannot be adjusted by a consistent correction to provide an equivalent to arterial concentrations. Estimates of insulin sensitivity, including $ISI_{Matsuda}$ and $ISI_{Cederholm}$, were also influenced by the sample method used, with venous blood providing higher values. The magnitude of the arterialised-venous difference was also greater after rest relative to after exercise, again preventing consistent corrections being applied.

These results also have implications when determination of absolute glucose concentrations is required, such as when assessing hypo- and hyperglycaemia. For example, peak postprandial plasma glucose concentrations and associated measures (i.e. time-to-peak) has implications for risk of metabolic disease and are often included as intervention outcomes (29). Our results demonstrate that 120 minute post-OGTT and peak plasma glucose concentrations are higher in arterialised relative to venous plasma. If suggested corrections are applied to obtain capillary concentrations from venous plasma (18), corrected venous glucose concentrations are greater than those measured in arterialised blood. As such, these corrections may need to be revisited.

It is beyond the scope of this work to establish the underlying mechanisms for the smaller arterialised-venous difference in glucose AUC after exercise compared to after rest. It is likely that the cycling induced localised insulin resistance in the

non-exercising forearm (20), potentially due to lipid deposition in non-exercise muscle (30). This may have reduced glucose uptake across the forearm, reducing the difference in concentrations between the arterialised and venous samples in the exercise condition. The smaller difference could also be explained by an increase in forearm blood flow post-exercise (31). Also of interest is the exploratory observation that participants with larger arterialised-venous differences for the plasma glucose and insulin AUC after rest, tended to have larger arterialised-venous differences for these outcomes after exercise. It is plausible that this may be attributable to differences between the participants in forearm muscle mass, and/or the anatomy of their vasculature, but this was not possible to ascertain from the current study.

To conclude, venous blood provides lower postprandial glucose concentrations and higher estimates of insulin sensitivity than arterialised samples, when common indices are applied. Moreover, the variation in plasma glucose concentrations between venous and arterialised samples is different after exercise compared to after rest, and thus corrections cannot readily be applied across all conditions. These results indicate that the method used for blood sampling (i.e. heating the hand for arterialised blood or sampling from an antecubital vein) is an important consideration when comparing studies that estimate glucose tolerance or insulin sensitivity from oral glucose loads.

REFERENCES FOR CHAPTER 4

1. Petersen KF, Dufour S, Savage DB, *et al.* (2007) The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci* 104(31),12587-94.

2. DECODE Study Group (2001). Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. *Arch Intern Med* 161(3), 397-405.

3. Belfiore F, Iannello S & Volpicelli G (1998). Insulin sensitivity indices calculated from basal and OGTT-induced insulin, glucose, and FFA levels. *Mol Genet Metab* 63(2),134-41.

4. Cederholm J & Wibell L (1990). Insulin release and peripheral sensitivity at the oral glucose tolerance test. *Diabetes Res Clin Pract* 10(2),167-75.

5. Matsuda M & DeFronzo RA (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22(9) 1462-70.

6. Stumvoll M, Mitrakou A, Pimenta W *et al.* (2000) Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes Care* 23(3), 295-301.

7. Gonzalez JT, Veasey RC, Rumbold PL *et al.* (2013) Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. *Br J Nutr* 110(4), 721-32.

8. Gonzalez J, Barwood M, Goodall S *et al.* (2015). Alterations in whole-body insulin sensitivity resulting from repeated eccentric exercise of a single muscle group: a pilot investigation. *Int J Sport Nutr Exerc Metab* 25(4), 405-10.

9. Chowdhury EA, Richardson JD, Holman GD *et al.* (2016) The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults. *Am J Clin Nutr* 103(3):747-56.

10. Hall R (1971). Vascular injuries resulting from arterial puncture or catheterization. *Br J Surg* 58(7):513-6.

11. Abumrad NN, Rabin D, Diamond MP *et al.* (1981). Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism* 30(9), 936-40.

12. Brooks DC, Black PR, Arcangeli MA *et al.* (1989). The heated dorsal hand vein: an alternative arterial sampling site. *JPEN J Parenter Enteral Nutr* 13(1), 102-5.

13. Liu D, Moberg E, Kollind M *et al.* (1992). Arterial, arterialized venous, venous and capillary blood glucose measurements in normal man during hyperinsulinaemic euglycaemia and hypoglycaemia. *Diabetologia* 35(3), 287-90.

14. McGuire E, Helderman J, Tobin J *et al.* (1976). Effects of arterial *versus* venous sampling on analysis of glucose kinetics in man. *J Appl Physiol* 41(4), 565-73.
15. Nauck M, Liess H, Siegel E *et al.* (1992) Critical evaluation of the 'heated-hand-technique'for obtaining 'arterialized'venous blood: incomplete arterialization and alterations in glucagon responses. *Clin Physiol* 12(5), 537-52.
16. Jensen MD & Heiling VJ (1991) Heated hand vein blood is satisfactory for measurements during free fatty acid kinetic studies. *Metabolism* 40(4), 406-9.
17. Sonnenberg G & Keller U (1982). Sampling of arterialized heated-hand venous blood as a noninvasive technique for the study of ketone body kinetics in man. *Metabolism* 31(1), 1-5.
18. World Health Organisation (1985) Diabetes Mellitus: Report of a WHO Study Group [meeting held in Geneva from 11 to 16 February 1985]. http://apps.who.int/iris/bitstream/10665/39592/1/WHO_TRS_727.pdf (accessed 9 August 2016)
19. Goodyear P, Laurie J, Kahn M *et al* (1998). Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* 49(1), 235-61.
20. Devlin JT, Barlow J & Horton ES (1989). Whole body and regional fuel metabolism during early postexercise recovery. *Am J Physiol Endocrinol Metab* 256(1), 167-72.
21. Brouns F, Bjorck I, Frayn KN *et al* (2005). Glycaemic index methodology. *Nutrition Research Reviews* 18, 145-171.
22. Jeukendrup A & Wallis G (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 26(1), 28-37.
23. Katz A, Nambi SS, Mather K *et al.* (2000). Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 85(7), 2402-10.
24. Levy JC, Matthews DR & Hermans MP (1998). Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care*, 21(12), 2191-2.
25. Jarrett R & Keen H (1976). Hyperglycaemia and diabetes mellitus. *Lancet*, 308 (7993), 1009-12.
26. Goldschmidt S & Light AB (1925). A method of obtaining from veins blood similar to arterial blood in gaseous content. *J Biol Chem* 64(1), 53-8.
27. Frayn K, Whyte P, Benson H *et al.* (1989). Changes in forearm blood flow at elevated ambient temperature and their role in the apparent impairment of glucose tolerance. *Clin Sci* 76(3), 323-8.

28. Dumke CL, Slivka DR, Cuddy JS *et al.* (2015). The effect of environmental temperature on glucose and Insulin after an oral glucose tolerance test in healthy young men. *Wilderness Environ Med* 26(3), 335-42.
29. Bonora E (2002). Postprandial peaks as a risk factor for cardiovascular disease: epidemiological perspectives. *Int J Clin Pract* 129, 5-11.
30. Schrauwen-Hinderling VB, Van Loon L, Koopman R *et al* (2003). Intramyocellular lipid content is increased after exercise in nonexercising human skeletal muscle. *J Appl Physiol* 95(6), 2328-32.
31. Green DJ, Bilsborough W, Naylor LH, *et al.* (2005). Comparison of forearm blood flow responses to incremental handgrip and cycle ergometer exercise: relative contribution of nitric oxide. *J Physiol* 562(2), 617-28.

RELEVANCE OF FINDINGS FOR THESIS

In **Chapter 4**, arterialised-venous differences for postprandial plasma glucose concentrations were higher after rest than exercise, so one consistent correction factor for blood glucose concentrations cannot be easily applied across these conditions. It is possible that the blood sample method used was also responsible for differences with results from **Chapter 3** *versus* previous work of glycaemia after exercise (15), as discussed prior to **Chapter 4**. Therefore, using data from **Chapter 4** and two-way (exercise or rest x time) ANOVAs the effect of using arterialised *versus* venous blood for detecting differences in postprandial blood glucose concentrations after exercise or rest was explored (**Figure 4.3**).

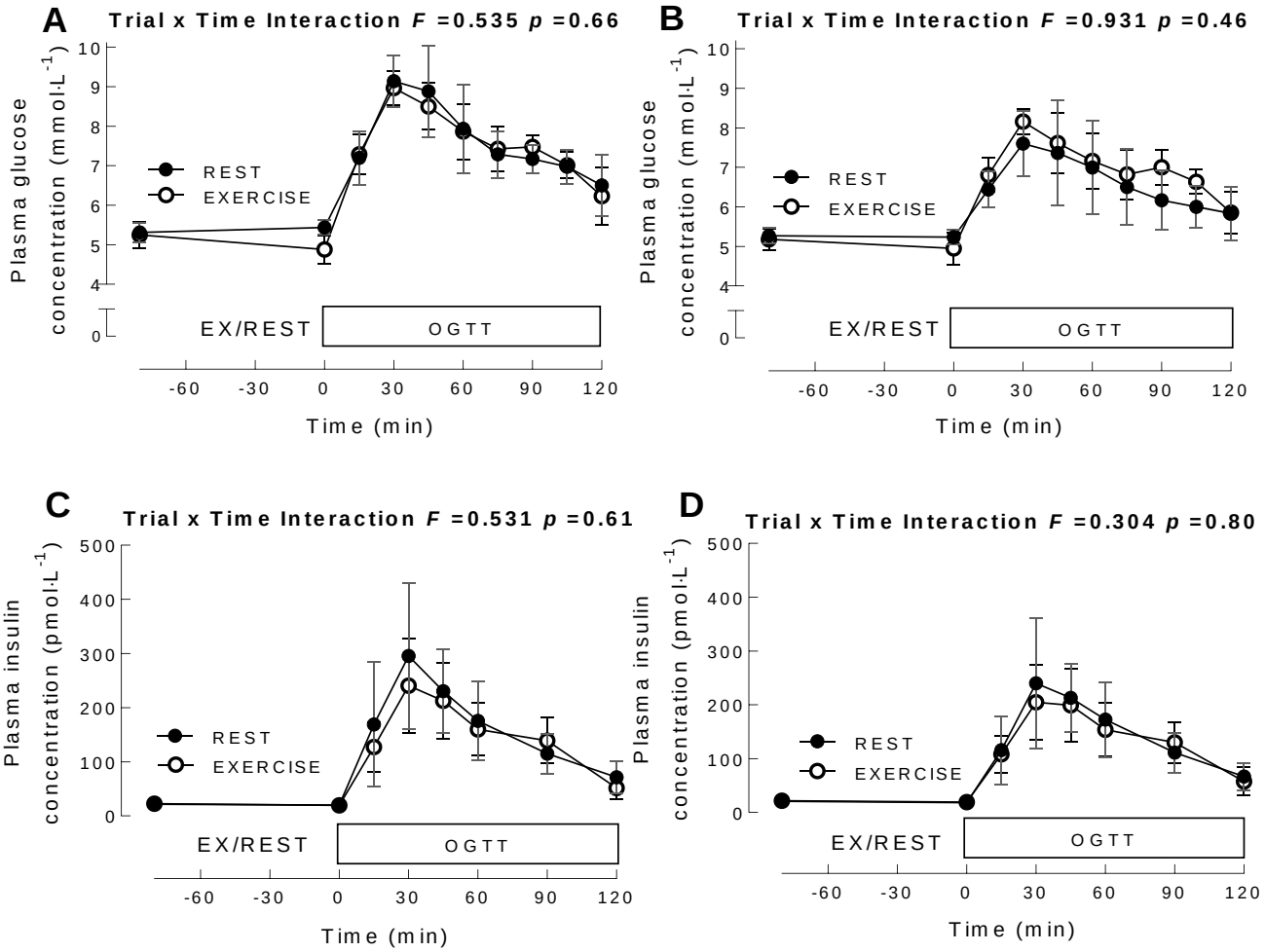

Figure 4.3. Plasma glucose (**A** [arterialised] and **B** [venous]) and plasma insulin (**C** [arterialised] and **D** [venous]) concentrations after rest or exercise. Data are means ± 95% confidence intervals; *n*=10 healthy men.

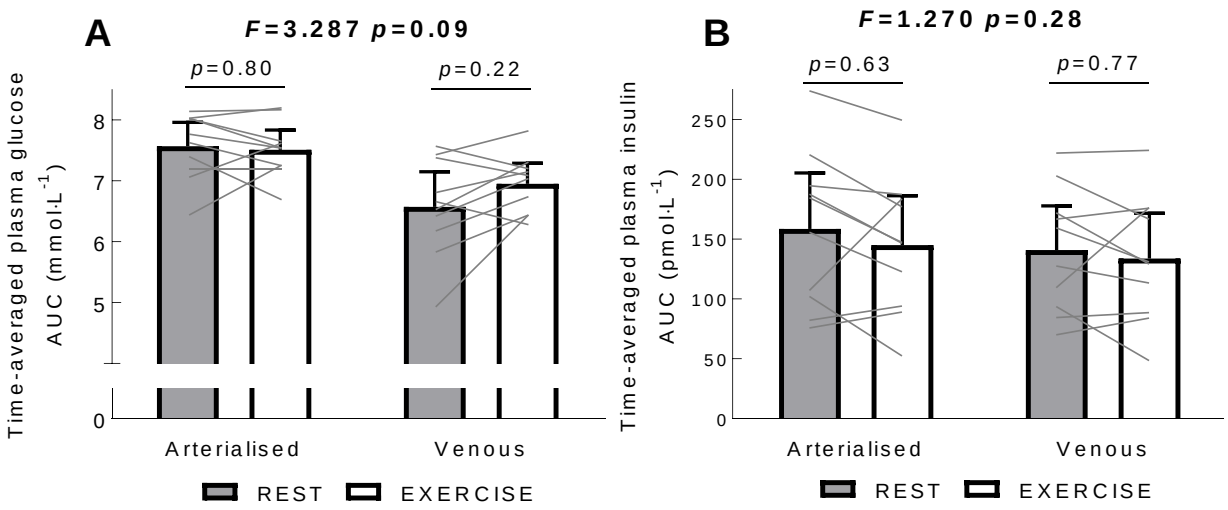

Figure 4.4. Plasma glucose AUC (**A**) and insulin AUC (**B**) after rest or exercise in arterialised and venous blood. Data are means ± 95% confidence intervals; *n*=10 healthy men.

These data are different to the results presented in **Chapter 4**, (where the main comparison was arterialised-venous differences with rest *versus* exercise) as they now show the differences between rest and exercise with arterialised *versus* venous blood. The aim of this sub-analysis was to establish whether a difference in postprandial glycaemia after exercise *versus* after rest would be clearer using venous compared to arterialised blood as this may explain the difference with results in **Chapter 3** and previous work (15). As shown in **Figures 4.3 and 4.4**, a significant difference in postprandial blood glucose concentrations after exercise *versus* rest was not apparent when using arterialised or venous blood samples. Nonetheless, it is possible that this analysis was not sufficiently powered to detect a difference between exercise and rest in this regard, as the sample method x trial interaction effect approached significance (*p*=0.09) and **Figure 4.4A** shows a trend for plasma glucose concentrations to be higher after exercise *versus* rest in venous blood (*p*=0.22 rest *versus* exercise) whilst this was much less clear in arterialised blood (*p*=0.80 rest *versus* exercise). The measurement of glucose in venous (*versus* arterialised) blood could thus explain the post-exercise second meal effect (i.e. higher postprandial blood glucose concentrations) in studies with prior fasted cycling *versus* fasting at rest. Lipid deposition in the non-exercising forearm may explain this result [and the lower glucose disposal rate in this tissue

at a meal consumed after fasted lower-limb exercise *versus* fasting at rest (431)] as discussed in **Chapter 4**. Finally, the work of Gonzalez *et al.* (15) where an increase in post-exercise postprandial blood glucose concentrations (in venous blood) was reported with breakfast consumption and exercise (*versus* breakfast at rest) but not fasting and exercise (*versus* fasting at rest) may be explained by the use of running instead of cycling exercise. This may alter blood glucose flux via splanchnic perfusion, intestinal damage (glucose appearance rates) and/or muscle damage (glucose disposal rates). More research is needed to investigate arterialised-venous differences for blood glucose concentrations in the context of these nutrient-exercise interactions and with different exercise modes.

In addition to sampling from a heated dorsal hand vein and an antecubital vein of the non-heated arm, blood was also sampled at the same time points from an antecubital vein of the heated arm in **Chapter 4**. In **Chapter 6**, the effects of exercise training before *versus* after breakfast for postprandial glycaemia was studied and arterialised blood sampled. However, cannulating a dorsal hand vein can be challenging in humans with obesity and this was not always achieved. In these instances, the cannula was placed in an antecubital vein of the heated arm and a correction was applied for plasma glucose concentrations during rest, using data in **Table 4.2 and Figure 4.3** (heated hand to heated arm). The magnitude of the difference between these sample sites was notable at ~ 0.80 $mmol \cdot L^{-1}$ for glucose at 30 min post-OGTT, in the context of the differences between groups for the pre- to post-intervention changes in glucose concentrations for the same OGTT time point in **Chapter 6** ([mean ± 95 % CI] 0.46 ± 0.70 control *versus* -0.56 ± 0.63 $mmol \cdot L^{-1}$ exercise). In **Chapter 4**, plasma insulin concentrations were not statistically different at any time point during the OGTT after rest or exercise in arterialised *versus* venous blood (**Table 4.3**). Non-significant differences were also less meaningful in the context of difference between groups for the pre- to post-intervention changes in insulin concentrations at the same OGTT time point in **Chapter 6** (80 ± 53 $pmol \cdot L^{-1}$ for control *versus* -185 ± 170 $pmol \cdot L^{-1}$ for exercise). A correction factor for sample methods was therefore not applied for plasma insulin concentrations in **Chapter 6** as differences with arterialised blood from a

heated hand and partially-arterialised blood from the heated arm would also likely be smaller in magnitude than differences with arterialised *versus* venous blood.

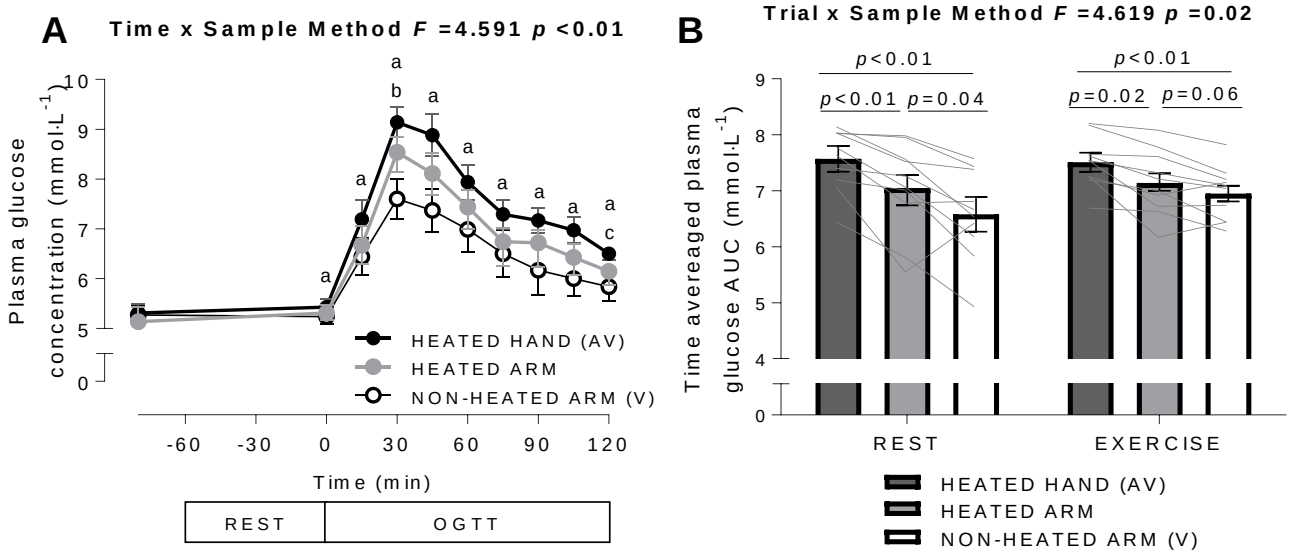

Figure 4.5. Plasma glucose concentrations during an oral glucose tolerance test (OGTT) after rest with arterialised blood (heated dorsal hand vein), partially-arterialised blood (antecubital vein of the heated arm) and non-areterialised blood (antecubital vein of the non-heated arm) (**A**) and the glucose AUC (**B**) after rest or exercise during morning fasting. Data are means ± normalised 95% confidence intervals for *n*=10 healthy men. In Panel **A** 'a' is a difference between heated-hand and non-heated arm, 'b' a difference between heated hand and heated arm and 'c' a difference between heated arm and non-heated arm with $p<0.05$.

Table 4.2. Plasma glucose concentrations from a heated hand, an antecubital vein from the heated arm and an antecubital vein from the non-heated arm during an oral glucose tolerance test during rest. Data are means and normalised 95% confidence intervals; 10 healthy men. * is a difference between sample methods with $p < 0.05$.

Time (min)	Heated hand ($mmol \cdot L^{-1}$)	Heated arm ($mmol \cdot L^{-1}$)	***Difference to heated hand*** ($mmol \cdot L^{-1}$)	Non-heated arm ($mmol \cdot L^{-1}$)	***Difference to heated hand*** ($mmol \cdot L^{-1}$)
0	5.43 (0.16)	5.31 (0.16)	***-0.12***	5.24 (0.14)	***-0.20 ****
15	7.19 (0.39)	6.67 (0.16)	***-0.52***	6.44 (0.37)	***-0.76 ****
30	9.14 (0.31)	8.54 (0.39)	***-0.60 ****	7.60 (0.40)	***-1.54 ****
45	8.88 (0.43)	8.11 (0.31)	***-0.77***	7.37 (0.43)	***-1.51 ****
60	7.94 (0.35)	7.44 (0.43)	***-0.49***	6.99 (0.45)	***-0.94 ****
75	7.29 (0.28)	6.74 (0.35)	***-0.55***	6.50 (0.48)	***-0.79 ****
90	7.17 (0.25)	6.72 (0.28)	***-0.44***	6.17 (0.49)	***-1.00 ****
105	6.97 (0.26)	6.43 (0.26)	***-0.55***	6.00 (0.35)	***-0.98 ****
120	6.50 (0.13)	6.15 (0.13)	***-0.35***	5.84 (0.28)	***-0.67 ****

4734

Table 4.3. Plasma insulin concentrations from a heated hand and an antecubital vein from the non-heated arm during an oral glucose tolerance test at rest. Data are means and normalised 95% confidence intervals; 10 healthy men.

Time (min)	Heated hand ($pmol \cdot L^{-1}$)	Non-heated arm ($pmol \cdot L^{-1}$)	***Difference to heated hand*** ($pmol \cdot L^{-1}$)
0	20 (1)	18 (1)	***-2***
15	169 (40)	116 (40)	***-53***
30	295 (25)	240 (25)	***-55***
45	230 (14)	213 (14)	***-17***
60	175 (6)	173 (6)	***-2***
90	115 (6)	111 (6)	***-4***
120	71 (6)	67 (6)	***-4***

CHAPTER 5 - BREAKFAST-EXERCISE & ENERGY BALANCE

Skipping breakfast before exercise creates a more negative 24-h energy balance: A randomized controlled trial in healthy physically active men.

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Sources of Support:
The European Society for Clinical Nutrition and Metabolism and The Rank Prize Funds funded this work. DT., JTG, and JAB are funded by the MRC (MR/P002927/1) and BBSRC (BB/R018928/1).

Trial Registration: https://clinicaltrials.gov/ [Number: NCT02258399]

Data Access Statement: The complete data set relating to this manuscript can be found at: https://researchdata.bath.ac.uk/610/

RELEVANCE TO THESIS

The results in **Chapter 3** showed that the metabolic and intramuscular responses to endurance-type exercise after breakfast cannot be inferred from the responses observed following exercise during morning fasting. Eating breakfast prior to exercise increased blood glucose disposal rates at a meal consumed after exercise, but this was offset by increases in the appearance rate of mainly meal-derived glucose. Consuming carbohydrate before exercise also increases blood glucose flux (272) and glucose concentrations (264, 270) as well as whole-body carbohydrate utilisation rates during exercise (264, 270), compared to when the exercise is performed during morning fasting. A difference in substrate utilisation and glycaemia during exercise with altered pre-exercise feeding may also have implications for the regulation of post-exercise energy balance. The concepts outlined here were introduced during **Chapter 1** of this thesis, but a more detailed discussion is now provided as context for the work in **Chapter 5**.

Numerous factors influence the regulation of energy balance and many of these are co-ordinated by neuroendocrine pathways in the hypothalamus (433, 434). Whilst not a primary focus of this thesis, it is proposed that afferents within the gastrointestinal (GI) tract can be stimulated by mechanical and chemical stimuli following meals, which may alter appetite and subsequent eating behaviours (435). For example, increasing mechanical stress in the stomach can decrease subsequent energy intake (436). This effect may also be potentiated by signals emitted after nutrients enter the GI lumen. Ghrelin is an orexigenic hormone that stimulates hunger and meal initiation when humans are fasting, and research in rodents also suggests that this effect may be mediated by its actions within the hypothalamus (437). During a 24-h fast, ghrelin secretion in healthy humans has peaks that align with expected meal times (438) and the administration of ghrelin *versus* saline in humans can stimulate an increase in energy intake (439, 440). Anorexigenic peptides, including glucagon-like peptide 1 (GLP-1), peptide tyrosine tyrosine (PYY), pancreatic polypeptide and oxyntomodulin can also be secreted from the GI tract (in humans) in response to nutrient intake and also alter subsequent eating behaviours, with research in rodents again highlighting a

mediating role for the hypothalamus (441). These GI-derived peptides are also secreted in response to the presence of specific nutrients in the intestine which, along with differences in their energy density and gastric emptying rates, explains why in humans, some foods have a greater satiating effect than others (442).

Leptin is a protein that is secreted from the adipose tissue, and in humans, blood leptin concentrations are positively correlated with fat mass (443). Leptin may have a role in the protection of further fat mass losses after longer-term diet or exercise induced energy deficits, which reduce blood leptin concentrations likely due to a loss of fat mass (444). A decrease in leptin concentrations following an energy deficit may be a mechanism to preserve fat stores, with leptin acting in a negative feedback loop, with the aim of maintaining the stability of adipose tissue. Fasting blood leptin concentrations are ~ 20-50 % lower after exercise training (340), but this response is less clear if additional food is provided (445). Lowering blood leptin concentrations decreases satiety and increases sensations of hunger in humans (446) and rodent research suggests that leptin acts by binding to the hypothalamic arcuate nucleus (447). If humans classified as overweight undergo weight loss, neural activity in brain regions relating to the regulatory, emotional and cognitive control of appetite are altered in response to external cues in a manner consistent with an increased desire to eat, yet this response can be ameliorated by leptin administration (448). Taken together, these studies provide convincing evidence of a hormonal regulation of energy balance in humans.

Energy balance may be regulated by negative feedback, whereby the induction of an energy deficit may stimulate behaviour change to restore energy balance (17). Changes to substrate balance may also contribute to a regulation of energy balance (351). Alterations to blood glucose flux [the glucostatic theory (449)] and/or glycogen availability [the glycogenostatic theory (352)] have both been proposed as possible regulators of energy balance. The glycogenostatic theory has received the most attention and suggests that because glycogen stores in the body have a smaller storage capacity than lipid stores [< 3000 kcal in a lean 75 kg male *versus* > 100,000 kcal (251)] they are more tightly regulated. As such,

the induction of a negative carbohydrate balance may stimulate energy intake and/or decrease energy expenditure to favour glycogen replenishment.

A number of studies in humans support a relationship between energy balance and whole-body carbohydrate balance. In one study, 24-h substrate utilisation in 67 men and 45 women was assessed using a respiratory chamber and *ad libitum* energy intake measured over the next 3 days (450). Carbohydrate utilisation (r=0.40; p<0.01) and carbohydrate balance (r=-0.34; p<0.01) over 24 h predicted subsequent energy intake. It has also been shown that trained people who often typically display a relatively low rate of carbohydrate utilisation during intensity-matched exercise are less likely to compensate for an exercise-induced energy deficit than untrained people (451, 452). Although demonstrating causality is difficult, this raises the possibility that substrate utilisation during exercise may be a regulator of post-exercise energy balance. In another study, healthy men cycled for 90 min at 60 % $\dot{V}O2_{peak}$, before an *ab libitum* lunch. The men who relied more heavily on carbohydrate as a substrate for exercise energy metabolism had a more positive net energy balance after lunch *versus* their resting trial, whereas the men with a lower rate of carbohydrate utilisation had a more negative energy balance *versus* their rest trial (453). However, the energy cost of exercise was also higher in men with a lower rate of carbohydrate use which contributed to that difference in energy balance. Correlations between carbohydrate use during exercise and energy intake post-exercise are reported elsewhere [r=0.76, p<0.01 (353) and r=0.57, p=0.02 (354)]. Nonetheless, in the first of those two studies all participants cycled at 90 W so the relative exercise intensity may have differed (and confounded the reported association) and in the second study, the exercise session was designed to expend 400 kcal for all of the participants, but the calculation was based on age-predicted HR and there were differences between the participants in the actual exercise-induced energy deficit of (mean ± SD) 445.2 ± 54.0. Despite this, research more specific to this thesis has shown that in healthy men, the energy deficit from breakfast omission prior to exercise is incompletely compensated for at lunch (15) or over 24 h (16). The lower 24-h energy intake with breakfast omission before exercise was not only attributable

to the breakfast, but to meals and snacks later in the day (16). That finding may also be explained by the smaller permutations to carbohydrate balance if exercise is performed during morning fasting compared to after breakfast.

Although the aforementioned studies have limitations and so must be interpreted cautiously, there is a considerable body of evidence to suggest that carbohydrate utilisation during exercise may regulate aspects of post-exercise energy balance. Whether the carbohydrate status of one store (i.e. muscle or liver glycogen) is a more potent regulator of energy balance is less clear. The post-exercise period is typically characterised by increased peripheral insulin sensitivity and glycogen synthase activity in exercised muscle (454). This directs ingested carbohydrates towards storage and reflects a biological drive to restore muscle glycogen. If the depletion of muscle glycogen does stimulate energy intake, one mechanistic link could be that cellular sensors such as AMPK convey information about glycogen availability to the hypothalamus (455). After exercise, AMPK activity is typically elevated in exercised skeletal muscle and increased further when the activity is performed with a reduced muscle glycogen content (313, 456). AMPK activity regulates aspects of metabolism in skeletal muscle (e.g. driving an increase in lipid utilisation) and AMPK also mediates the effects of adipocyte- or GI-derived hormones in the hypothalamus (457, 458). In mice, fasting increases (and feeding inhibits) AMPK activity in the hypothalamus and if adenoviruses expressing active AMPK are injected into the basal medial hypothalamus of mice, hypothalamic AMPK activity increases and is associated with an increased energy intake and gains in body mass (458). However, if mice perform exercise hypothalamic AMPK activity is not always increased (459), so any link between AMPK activity and the regulation of energy balance after exercise is currently not clear.

Interleukin-6 (IL-6) is a peptide that is secreted from active skeletal muscle during exercise (248). IL-6 production may also be modulated by the glycogen content of exercising muscles (460), leading to speculation that IL-6 operates as a cellular energy sensor (461). IL-6 increases lipolysis in the adipose-tissue and stimulates lipid utilisation (77). However carbohydrate ingestion blunts increases in plasma

IL-6 concentrations during exercise (461). As such, if substrate utilisation during exercise does regulate energy balance responses post-exercise, IL-6 secretion may help to explain this association, with a link to muscle glycogen. Whole-body IL-6 knockout mice appear to be susceptible to obesity and show increased fat masses (462). In a human study, men and women with obesity performed 12 weeks of endurance-type exercise, with or without infusions of an IL-6 receptor antagonist, tocilizumab. This study suggested that IL-6 signalling was required for exercise training to reduce visceral fat mass, because when tocilizumab was administered, the exercise-induced decrease in visceral fat mass was diminished (463). Nonetheless, these findings may be best interpreted as evidence of a role for substrate utilisation *per se* during exercise in the regulation of energy balance as opposed to a mechanistic link between muscle glycogen status and energy balance. This is because evidence of an association between plasma IL-6 levels and post-exercise appetite is lacking (464) and because low muscle glycogen concentrations would be expected to increase IL-6 secretion and stimulate lipid utilisation, thereby decreasing carbohydrate utilisation. This would then, in theory, decrease the likelihood of compensation after exercise, which is in contrast to suggestions that lowering muscle glycogen concentrations increases subsequent energy intake. In addition to a lack of a clear mechanistic link between muscle glycogen status and behaviours that contribute to energy balance after exercise, large reductions in muscle glycogen concentrations (~ 50 %) achieved with diet and exercise does not always stimulate a clear increase in energy intake (465).

Large variance in liver glycogen availability may be more robustly defended than muscle glycogen because liver glycogen is a substantive source of glucose for the systemic circulation and for vital organs including the brain during fasting (30). A manipulation of whole-body carbohydrate balance via diet modifications over 1-2 days using isoenergetic diets but with varying fat and carbohydrate contents does not substantially change subsequent energy intake responses (466, 467). However, in a longer study, a low, moderate or high fat diet (20 %, 40 % or 60 % of kcal) was consumed *ad libitum* for a week and an association between the subsequent carbohydrate balance and energy intake on the next day was shown

(467). If dietary carbohydrate is restricted, the liver glycogen content is decreased due to increased glycogenolysis, to help maintain blood glucose concentrations via increased HGP, especially as muscle glycogen does not contribute to blood glucose maintenance due to a lack of glucose-6-phosphatase (468). Despite this, with prolonged dietary carbohydrate restriction, the muscle glycogen content is likely to also be decreased, as muscle glycogen is utilised during physical activity and not restored unless carbohydrate is ingested (303). Therefore, investigating whether liver glycogen has a specific role in the regulation of energy balance in humans is difficult, because it is hard to manipulate liver glycogen status over longer than a single overnight fast without also altering muscle glycogen status. Nonetheless, in mice, a link between liver glycogen status and energy balance has been shown. Mice that have high levels of liver glycogen show protection from body mass gains, which was attributed to a reduction in energy intake (469). Furthermore, mice that overexpress *hepatic protein targeted to glycogen*, which increases liver glycogen concentrations by both activating glycogen synthase and inhibiting glycogen phosphorylase, show a lower energy intake, increased energy expenditure and lower subcutaneous fat mass, compared to wild type mice (355).

A plausible link between liver glycogen status and alterations in energy balance behaviours is that signals about energy availability from the liver are transmitted to the brain via the hepatic vagus nerve. In support of this, when hepatic branch vagotomy (HBV) was performed on mice overexpressing *hepatic protein targeted to glycogen*, HBV blunted the effect of increased liver glycogen accumulation on energy intake, which was apparent when sham operations were performed (355). This was explained by the theory that a higher liver glycogen content maintains hepatic ATP levels which is a signal of energy availability and inhibits the firing of hepatic vagal afferents and an activation of the nucleus tractus solitarius resulting in a suppression of appetite and a decrease in energy intake (470). However, HBV operations can also inhibit the innervation of parts of the GI tract that may alter the secretion of appetite-regulating peptides, which somewhat complicates any interpretation of those mechanistic results (470). It is also possible that liver glycogen status may mediate appetite and energy balance behaviours via

changes in endocrine signalling. Specifically, fibroblast growth factor 21 (FGF21) is a protein primarily produced by the liver and is responsive to alterations in liver carbohydrate status. In rodents carbohydrate ingestion increases hepatic *FGF21* mRNA expression and plasma FGF21 concentrations (471) and the increase in plasma FGF21 concentrations can be associated with a reduction in meal size, a reduction in sweet-seeking behaviours and decreases in body mass (471). This response also seems to be enhanced with fructose-containing sugars, providing a possible link to the liver because fructose-containing sugars can increase liver glycogen synthesis to a greater extent than glucose (471). However, in humans there is currently limited evidence linking hepatic glycogen availability or FGF21 to energy balance behaviours, so more research is warranted.

Finally, as discussed previously, leptin is an important hormone in the regulation of appetite and energy balance in humans. An acute (~ 3 day) decrease in carbohydrate availability and 4 weeks of a low carbohydrate diet (relative to when an isocaloric high carbohydrate diet is consumed) lowers fasting plasma leptin concentrations (472, 473). An acute bout of exercise typically decreases glucose uptake in the adipose tissue (474), which may be important as leptin secretion from the adipose tissue can be altered depending on blood glucose availability [e.g. infusing glucose at ~ 4 $g \cdot h^{-1}$ prevents a fall in leptin during periods of fasting (475)]. Exercise also increases AMPK activation within the adipose tissue (476), which may supress leptin secretion via mTOR signalling (477). Despite this, whilst the consumption of a modest amount of carbohydrate before exercise alters metabolism during exercise, it does not clearly alter the exercise-induced change in the mRNA expression or the content of AMPK in the adipose tissue *versus* exercise in the fasted-state (14). However, as hyperinsulinemia stimulates leptin secretion (478), any link between carbohydrate utilisation during exercise and plasma leptin concentrations may be explained by plasma insulin concentrations (472). Overall, if carbohydrate utilisation during exercise is indeed a regulator of post-exercise energy balance in humans, the current evidence suggests that this relationship may be explained by changes to the liver glycogen content (i.e. liver glycogen utilisation rates) and/or plasma leptin concentrations.

One of the limitations with the previous human studies investigating whether liver or muscle glycogen availability is a more important regulator of energy balance, is the absence of direct glycogen measurements, with inferences made only from estimations of substrate balances at a whole-body level [where a positive carbohydrate balance favours carbohydrate storage as glycogen and a negative carbohydrate balance favours net glycogen utilisation (479)]. As such, to provide further insights into the tissue-specific regulation of post-exercise energy balance in **Chapter 5**, substrate balances were assessed using indirect calorimetry and researcher-weighed meals, but were also combined with the glucose flux data from **Chapter 3**. As such, during exercise the utilisation of carbohydrate derived from the liver *versus* skeletal muscle was estimated. Plasma glucose utilisation was assumed to be equivalent to the plasma glucose rate of disposal, as this has been confirmed previously (480). As blood glucose utilisation during fasted-state exercise is almost completely derived from the liver (30), an estimation of liver glucose utilisation was made. Then, by subtracting this value from whole-body carbohydrate utilisation rates, muscle glycogen utilisation was also estimated.

This estimation of blood glucose disposal rates has limitations if tracer: tracee steady-state is not achieved, but this can be avoided by using non-steady-state modelling and frequent blood sampling (399). The protocol used in **Chapter 3** was designed to assess glucose flux post-exercise, so blood was sampled more infrequently during exercise. Despite this, the blood glucose appearance values of (mean ± SD) of 3.75 ± 1.45 $mg \cdot kg^{-1} \cdot min^{-1}$ in **Chapter 3** do not clearly differ to values from research assessing liver glycogen utilisation during exercise with ^{13}C magnetic resonance spectroscopy (MRS) and glucose tracer methods (481). One potential limitation with the tracer method that was used in **Chapter 3** to estimate muscle glycogen utilisation is that whole-body carbohydrate utilisation rates (from which this measure was partly derived) can be altered by any utilisation of ketone bodies such as *β*-hydroxybuturate (BHB). However, with the concentrations of BHB that we report in **Chapter 3**, the utilisation of BHB (482) and the effect on carbohydrate utilisation rates (88) is likely to be negligible. Another consideration is that whole-body carbohydrate utilisation rates reflect not only plasma glucose

and muscle glycogen utilisation, but also the utilisation of lactate. In **Chapter 3,** plasma lactate concentrations for fasted-exercise were 2.79 ± 1.00 mmol·L^{-1}. With similar blood lactate concentrations during fasted cycling in healthy men, lactate made a contribution to whole-body carbohydrate utilisation of ~ 25 % (483). In that study, further increasing plasma lactate concentrations resulted in a higher rate of lactate utilisation (from 7 to 9 mg·kg^{-1}·min^{-1}) but this seemed to be almost completely accounted for by a decrease in plasma glucose utilisation, measured via tracer methods. As such, it is unlikely that the estimation of muscle glycogen utilisation during exercise in **Chapter 5** would have been meaningfully influenced by the absence of a measure of lactate utilisation, even if this did differ across participants, as blood glucose utilisation was assessed and accounted for in the estimations of muscle glycogen utilisation.

Alternative methods of assessing glycogen utilisation also have limitations. The measurement of glycogen concentrations has historically involved the chemical analysis of biopsy samples (268). However, if measuring glycogen in this way, the measured concentration is only representative of the tissue that is sampled and the site of sampling within the muscle. The biopsy procedure is also invasive and can induce a catecholamine response and influence the concentrations of various blood metabolites in unaccustomed participants (484). Obtaining a liver biopsy sample is also difficult and is rarely performed outside of a clinical setting (485). A non-invasive alternative to biopsy derived measures of muscle or liver glycogen is ^{13}C MRS, which measures the magnitude of a signal corresponding to the quantity of 1-^{13}C glucose in the glycogen-bound form. Some nuclei are electrically charged, so if a magnetic field is applied using a probe positioned on skin adjacent to a muscle of interest, an energy transfer occurs at a wavelength corresponding to a specific radio frequency. When the nuclei spin returns to its basal level, energy is then emitted at the same frequency. A signal that matches this frequency is measured in order to yield an MRS spectrum for the nucleus concerned. One limitation of this method for measuring glycogen is that only ^{13}C is detected, as the nuclei of ^{12}C do not possess the magnetic moment required to align with the magnetic field. Thus, the diet of the participants can alter glycogen

measurements, as dietary carbohydrates have different $^{13}C/^{12}C$ enrichments. However, these differences in ^{13}C abundance are small relative to changes in the liver and muscle glycogen content with exercise (486), so can likely be neglected as a confounding factor for glycogen measurements with this method. However, a measure of glycogen concentrations before and after exercise only provides net- and not absolute-rates of glycogen utilisation, which may be important as glycogenesis can occur during exercise (487). As such, the tracer methods used in **Chapter 5** are appropriate for measuring absolute rates of glycogen utilisation.

Another limitation with previous human studies assessing the effect of differences in substrate utilisation during exercise on post-exercise energy balance, is the absence of measures of subsequent free-living energy expenditure. Indeed, most studies have only measured energy intake and/or energy expenditure, but in a controlled environment. During periods of fasting, compensatory reductions in free-living energy expenditure can occur and this has been attributed to a reduction in non-exercise physical activity (328). In lean adults, extending an overnight fast to midday decreases morning and 24-h physical activity energy expenditure compared to when breakfast is consumed (328). In humans with obesity, physical activity is also lower during the morning with breakfast omission *versus* consumption (329). It is therefore surprising that an assessment of energy expenditure outside of a controlled laboratory environment has never been completed with exercise and breakfast omission *versus* consumption. Thus, one of the aims of **Chapter 5** was to investigate whether breakfast consumption *versus* omission before exercise alters post-exercise energy intake *and* energy expenditure over 24 h. A second aim was to investigate the role of muscle or liver glycogen utilisation during exercise in regulating post-exercise energy balance.

This declaration concerns the article entitled:			
Skipping breakfast before exercise creates a more negative 24-h energy balance: A randomized controlled trial in healthy physically active men.			
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]			
Publication details (reference)	Edinburgh, R. M., Hengist, A., Smith, H. A., Travers, R. L., Betts, J. A., Thompson, D., Walhin, J-P., Wallis, G.A., Hamilton, L.D., Stevenson, E. J., Tipton, K.D. & Gonzalez, J.T. (2019). Skipping breakfast before exercise creates a more negative 24-h energy balance: A randomized controlled trial in healthy physically active men. *Journal of Nutrition, 149* (8), 1326-1334.		
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Candidate's contribution to the paper (provide details, and also indicate as a percentage)	**Formulation of ideas:** RME, JTG, KDT, DLH, EJS, JAB and DT designed the research **Design of methodology:** RME, JTG, KDT, DLH, EJS, JAB and DT designed the methodology. **Experimental work:** RME (> 90 %), JTG, AH, HS, RLT and J-PW conducted the research and RME analysed the data. RME was present for the data collection on trial days, prepared and analysed blood samples (for metabolites and hormones), completed data and statistical analysis and was heavily involved in all aspects of the experimental work. **Presentation of data in journal format:** RME (> 80 %) and JTG wrote the paper, and all authors contributed to earlier versions of the manuscript and approved the final version of the manuscript.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	R.M. Edinburgh	**Date**	2nd July 2019

5077

ABSTRACT

Background: At rest, breakfast omission lowers daily energy intake, but also lowers energy expenditure, attenuating any effect on energy balance. The effect of breakfast omission, on energy balance when exercise is prescribed, is unclear. **Objective:** The aim was to assess the effect of pre-exercise breakfast omission *versus* consumption on 24-h energy balance. **Methods:** Twelve healthy active young men (age 23 ± 3 years, body mass index 23.6 ± 2.0 $kg \cdot m^{-2}$) completed three trials in a randomized order (separated >1 week): a breakfast of oats and milk (431 kcal; 65 g CHO, 11 g FAT, 19 g PRO) followed by rest (BR); breakfast before exercise (BE; 60 min cycling at 50% peak power output); and overnight-fasting before exercise (FE). The 24-h energy intake was calculated from breakfast, and an *ad libitum* lunch, snacks and dinner. Indirect calorimetry with heart-rate-accelerometry were used for substrate utilization and 24-h energy expenditure. A [6,6-$^{2}H_{2}$]-glucose infusion was used to investigate tissue-specific carbohydrate utilization. **Results:** Energy balance (24 h) was -400 kcal (normalized 95 % confidence intervals: -230 to -571 kcal) with exercise and breakfast omission (FE), and this was significantly lower than the rest trial (BR; 492 [332 to 652] kcal) and also the exercise but with prior breakfast consumption (BE; 7 [-153 to 177] kcal; both $P<0.01$ *versus* FE). Plasma glucose utilization in FE (mainly representing liver glucose utilization), was positively correlated with energy intake compensation at lunch ($r= 0.62$, $P= 0.03$), suggesting a role for liver carbohydrate use in post-exercise energy balance regulation. **Conclusion:** Neither exercise energy expenditure nor the restricted energy intake via breakfast omission were completely compensated for post-exercise. In healthy men, pre-exercise breakfast omission creates a more negative daily energy balance and could therefore be a useful strategy to induce a short-term energy deficit.

INTRODUCTION

Obesity is an escalating global epidemic, and is a consequence of a chronic positive energy imbalance. In addition to reducing calorie intake, regular exercise is a commonly proposed strategy for facilitating weight loss or weight maintenance (1). This is because exercise increases energy expenditure and thus alters energy balance, thereby potentially favoring the conditions for reductions in body and fat mass. Despite this, exercise training interventions often report smaller than expected fat and body mass losses (2,3). This modest response can be explained by compensation of energy balance behaviours (either the activity stimulating energy intake and/or decreasing physical activity outside of the prescribed exercise), and this reduces the energy deficit created by the exercise energy expenditure (4). A similar example of this compensation, is that breakfast omission at rest (i.e. reduced energy intake) decreases morning non-exercise physical activity in lean humans and humans with obesity (5,6).

In particular, altering carbohydrate balance may have implications for subsequent energy balance (7). Endogenous carbohydrate stores (liver and muscle glycogen) have a smaller storage capacity than lipid stores [< 3000 kcal in a lean 75 kg male *versus* > 100,000 kcal for lipids (8,9)]. Due to this limited storage capacity, the Glycogenostatic theory (7) proposes that endogenous carbohydrate stores are closely regulated, and because of this, glycogen depletion may increasingly stimulate compensatory energy intake in order to favour the replenishment of these stores. For example, in humans there is some (albeit limited) evidence that individuals who display high(er) rates of carbohydrate utilization when exercising are more likely to compensate with a higher post-exercise energy intake (8,10,11). Whilst short-term (1-3 days) alterations in glycogen availability with exercise or diet does not always result in measurable compensation in energy intake (8), mice overexpressing hepatic protein targeted to glycogen (which increases liver glycogen concentrations) also display reduced energy intake and increased energy expenditure (12). If carbohydrate metabolism is indeed a driver of subsequent energy intake, any strategies that attenuate carbohydrate use during exercise may help protect against compensation through post-exercise

energy intake (thereby reducing any erosion of the exercise-induced energy deficit). One strategy that reduces the rate of whole-body carbohydrate utilization during exercise is pre-exercise fasting (i.e. breakfast omission) (13). Furthermore, since breakfast omission under resting conditions does not result in energy intake compensation at lunch (14,15) any potential protection against a subsequent higher energy intake (by a lower carbohydrate utilization during exercise when fasting) is unlikely to be compromised due to the lower pre-exercise food intake (i.e. with the omission of breakfast).

If moderate intensity (55% $\dot{V}O_2$peak) endurance-type exercise is performed in the overnight fasting state, whole-body carbohydrate utilization is lower (and fat utilization is higher) compared to exercise after breakfast (13). Interestingly, humans do not fully compensate with energy intake at a subsequent meal if breakfast is omitted before exercise (15,16), which extends observations made with breakfast consumption during resting conditions (5). These findings are consistent with a role for carbohydrate balance in the regulation of energy balance. However, an objective assessment of *daily energy balance* (when energy intake *and energy expenditure* are assessed in a free-living setting) after exercise with prior breakfast consumption *versus* omision has never been conducted. This limitation is especially important in light of findings that the omision of breakfast at rest decreases sedentary and light (i.e. non-exercise) physical activity (5,6). Whether the carbohydrate status (content or rate of utilization) of a specific tissue (liver or muscle glycogen) is a more potent regulator of post-exercise energy balance also remains unknown. Indeed, no study has specifically assessed a relationship between muscle or liver carbohydrate utilization and post-exercise energy balance in humans. Investigating this response would provide insights into the regulation of post-exercise energy balance, and this information could be applied to refine exercise and nutritional strategies (e.g. pre-exercise fasting to lower liver glycogen concentrations *versus* exercise to deplete muscle glycogen).

Therefore, the aim of this experiment was to investigate the role of carbohydrate availability during exercise on net energy balance over 24 h. In addition, the application of glucose tracer methods (to assess hepatic glucose output and utilization) combined with indirect calorimetry, was implemented to address a secondary aim of exploring the tissue-specific regulation of energy balance.

METHODS

Ethical Approval

These results were collected as part of a wider study (17), but none of the data have been previously published. The study was completed at the University of Bath (Bath, UK) as per the Declaration of Helsinki. Ethical approval was given by the National Health Service South-West Research Ethics Committee (15/SW/0006). The trial was registered at clinicaltrials.gov (NCT02258399). Prior to participation, written and informed consent was obtained from all participants.

Study Design

This study adopted a randomized cross-over design (randomization by JTG using Research Randomizer version 3.0, http://www.randomizer.org/). Preliminary testing was followed by three trials (7-30 d apart), which were breakfast followed by rest (BR), breakfast followed by exercise (BE) and overnight fasting followed by exercise (FE), in a random and counterbalanced order. A protocol schematic is shown in **Figure 1**. For all trials participants arrived at the laboratory at 0800 ± 1 h having fasted overnight (12-14 h). In BR, upon arrival at the laboratory, a porridge breakfast was consumed, followed by 3 h of rest, and a 2-h oral glucose tolerance test (OGTT). In BE, the breakfast was consumed, before 2 h rest and 60 min of cycling, and the OGTT. In FE, breakfast was omitted but the trial otherwise replicated BE. After the OGTT, participants were provided an *ad libitum* lunch (within-lab) and a researcher-weighed food package, for consumption over the remaining 24-h trial (free-living). Daily energy expenditure was assessed via indirect calorimetry (for within-laboratory components) and heart-rate with accelerometry (free-living after leaving the laboratory). All trials were completed in similar laboratory conditions as previously described (17).

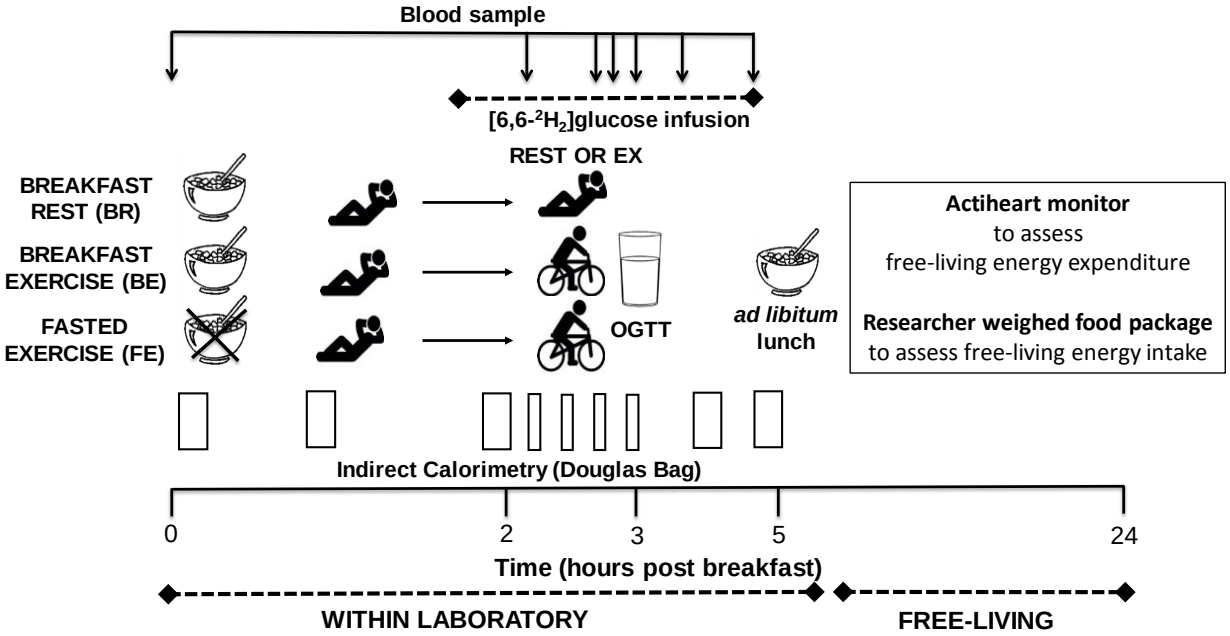

Figure 1. Schematic. Twelve healthy young, physically active men completed three trials in a randomized order: breakfast followed by rest (BR), breakfast followed by exercise (BE), or overnight fasting followed by exercise (FE). Daily energy intake was determined by an *ad libitum* lunch, snacks and dinner meals. Indirect calorimetry (within-lab) and heart-rate-accelerometry (free-living) were used to assess substrate use and daily energy expenditure. A [6,6-$^{2}H_2$]-glucose infusion was used to assess tissue-specific carbohydrate utilization.

Participants

Twelve healthy and physically active men were recruited from Bath (UK) and the surrounding area, between May and November 2015. Participant characteristics are shown in **Table 1**. The main exclusion criteria for participants included them having a history of metabolic disease, or any condition that may have posed undue personal risk to the participant or introduced bias to the study. Participants were also asked to confirm that they were not taking any medication that may have influenced any of the reported outcomes. The participant's stature was measured to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK) and their body mass was recorded to the nearest 0.1 kg using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). A whole-body dual energy x-ray absorptiometry scan was completed to quantify fat and fat-free mass (DEXA; Discovery, Hologic, Bedford, UK). An incremental exercise test was completed on an electronically-braked ergometer (Excalibur Sport, Lode® Groningen) (17).

Table 1. Participant Characteristics. Values are means ± SDs, *n*=12.

Age (y)	23 (3)
Stature (cm)	179.8 (4.4)
Body mass (kg)	76.3 (7.9)
Body Mass Index ($kg \cdot m^{-2}$)	23.6 (2.0)
Fat mass (kg)[1]	10.6 (3.7)
Fat Mass Index ($kg \cdot m^{-2}$)[1]	3.26 (1.12)
Body fat (%)[1]	14 (4)
Fat free mass (kg)[1]	65.5 (6.4)
Resting metabolic rate ($kcal \cdot day^{-1}$)	2091 (101)
$\dot{V}O_2$peak ($L \cdot min^{-1}$) *	4.00 (0.72)
$\dot{V}O_2$peak ($mL \cdot kg \cdot min^{-1}$) *	53 (10)
Peak Power Output (W)	317 (67)
HR_{MAX}, ($beats \cdot min^{-1}$)	189 (10)

$\dot{V}O_2$peak = peak oxygen uptake. **n* = 11, due to technical difficulties with the breath-by-breath analysis during one preliminary testing session. [1] = by DEXA.

Main Trials

Participants abstained from alcoholic and caffeinated drinks for 24 h prior to all trials. Food intake ceased at 2000 h on the evening before trials and participants then fasted overnight (minimum 12 h), consuming only water (*ad libitum*) during this period. The final meal consumed by the participants on the evening before all trials was provided (Spinach and Ricotta Cannelloni [Tesco]) to ensure the energy and macronutrient intake across participants was standardized (592 kcal of energy ([2,479 kJ]; 51 g CHO, 32 g FAT, 25 g PRO). Participants were also asked to refrain from strenuous physical activity for 24 h prior to trials, but were allowed to otherwise maintain their normal physical activity behaviours (replicated for subsequent trials). To help ensure this standardization, participants recorded an activity diary and wore a physical activity monitor (Actiheart™; Cambridge Neurotechnology, Papworth, UK). There were no differences between trials in

pre-trial physical activity energy expenditure as previously reported (17). Participants arrived at the laboratory at 0800 ± 1 h and this time was replicated for subsequent trials. They voided and all further urine samples were collected for urine urea concentrations and estimations of urinary nitrogen excretion. An intravenous catheter (BD Venflon Pro, BD, Helsingborg, Sweden) was placed retrograde into a dorsal hand vein that had been heated for 20 min using a heated-air box set to 55°C. A baseline blood sample (10 mL) was drawn, before a 5-min expired gas sample was collected. In BE and BR, a porridge breakfast was then consumed, but in FE participants were only permitted water. This was followed by 2 h of rest (activities such as reading whilst semi-recumbent were allowed), with 5-min expired gas samples collected hourly. During this period, a catheter was inserted into an antecubital vein and a primed variable-rate infusion of [6,6-$^{2}H_{2}$]glucose was initiated. After the 2-h rest period (in BE and FE only), participants began 60 min of cycling at 50 % peak power output (PPO) on an ergometer (Lode Corival, Lode B.V, Groningen, Netherlands). In BR, participants continued to rest during this period. Expired gases were collected every 15 min for 2 min and blood was sampled at 40 and 50 min of exercise (rest in BR). Then a 2-h OGTT was completed, with blood sampled every 10 min and 5-min expired gas samples collected hourly. Muscle biopsy samples were collected pre-and post-OGTT as detailed elsewhere [17]. Participants were given a researcher-weighed *ad libitum* lunch. After lunch, participants left the laboratory with a researcher-weighed food package and wore an Actiheart™ for the remainder of the 24-h trial.

Test meals

Prior to participation participants confirmed they had no allergies or aversions to any of the foods provided. The breakfast was 72 g of instant refined oats (Oatso Simple Golden Syrup, Quaker Oats) and 360 mL of semi-skimmed milk (Tesco), providing 431 kcal of energy ([1803 kJ]; 65 g CHO, 11 g FAT, 19 g PRO). Lunch was oats (Everyday Value® Tesco), whole milk (Tesco), maltodextrin, whey protein isolate (both Myprotein, Northwich, UK), olive oil (Tesco), and water, designed to limit the degree of palatability, with the aim of preventing

overconsumption (18). The meal provided 150 kcal of energy per 100 g of cooked food ([626 kJ]; 20 g CHO; 5 g FAT; 5 g PRO) and was terminated when participants said that they felt 'comfortably full'. Fresh, warmed portions were continually provided to ensure that the participant finishing a portion was not responsible for meal termination. Participants ate the lunch meal in isolation and every attempt was made to avoid cues that may have influenced their eating behaviors (the television was switched off and their mobile phones were not present during the meal). Remaining food was removed and weighed out of the sight of participants. The free-living food package comprised: [1] a pasta meal, of pasta, tomato sauce, cheddar cheese and olive oil (prepared by the researchers), providing 151 kcal of energy per 100 g of cooked food ([632 kJ]; 20 g CHO; 6 g FAT; 5 g PRO) [2] 4 x 35 g snack bars (GoAhead®; 367 kcal [1536 kJ] per 100 g; 74 g CHO, 8 g FAT; 3 g PRO) and [3] 2 x 180 mL chocolate milk flavor drinks (Mars® Milk; 63 kcal [264 kJ] per 100 mL; 10 g CHO; 2 g FAT; 3 g PRO). Participants were instructed to eat until they were 'comfortably full', not to eat or drink anything not provided by this food package (water was *ad libitum*) and to bring any remaining food to the laboratory the next morning. The carbohydrate, fat and protein intake was taken as grams provided minus grams remaining.

Blood sampling and analysis

Whole blood was dispensed into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) which were centrifuged (4°C and 3500 *g*) for 10-min (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK) for plasma. This was dispensed into 0.5 mL aliquots and frozen at -20°C, before longer-term storage at -80°C. Plasma glucose concentrations (intra-assay coefficient of variation [CV], 3.2 %; inter-assay CV 3.8 %) were measured on an automated analyzer (Daytona; Randox Lab, Crumlin, UK). Plasma leptin and FGF21 concentrations were measured with commercially available ELISAs (Mercodia AB, Uppsala, Sweden; intra-assay CV 5.8 %, inter-assay CV 7.1 % for leptin and BioVendor Research & Diagnostic Products; Karasek, Czech Republic; run in singular for FGF21). Blood samples were analyzed in batch after all sample collection was completed, and for a given participant all samples were run on the same plate.

Plasma [$^{2}H_{2}$]-glucose enrichments were determined via gas chromatography-mass spectrometry as described elsewhere [17] (GC, Agilent 6890N; MS, Agilent 5973N; Agilent Technologies, Stockport, UK). Radziuk's two-compartment non-steady state model was used to assess plasma glucose flux (19, 20).

Energy expenditure and substrate utilization

Indirect calorimetry was used to assess energy expenditure and substrate utilization from expired gas samples (21). An average of the baseline expired gas sample from the trials was used for resting metabolic rate. Urinary nitrogen excretion was estimated from urine urea concentrations, which were measured on an automated analyzer (Daytona; Randox Lab, Crumlin, UK). This allowed protein utilization to be accounted for in calculations of carbohydrate and lipid utilization. Free-living physical activity energy expenditure was assessed (from when the participant left the laboratory until 24 h post breakfast consumption [or omission]) using an Actiheart^{TM}, which integrates accelerometer and HR signals (22). Physical activity energy expenditure was calculated using branched-equation modelling (23) with measured energy expenditure and heart rate values from exercise and from rest entered into the ActiheartTM software for an individually calibrated model (24,25). Data were considered to be of a useable quality if >90 % of the activity trace was 'not lost' with <30 % of the HR trace extrapolated by the software. Daily energy expenditure was calculated from indirect calorimetry (for within-lab), and the Actiheart^{TM} data (for free-living).

Calculations and statistical analysis

A sample size calculation was completed based on previous research, where a difference in energy balance (in-lab) between a FE and BE trial was (mean ± SD) 1580 ± 1410 kJ (15). With an α-level of 0.05, 12 participants were required to provide an 80% chance of detecting a statistically significant difference in energy balance with a repeated measures ANOVA and 3 trials. The total (AUC) and incremental (iAUC) area under the concentration-time curves for plasma FGF-21 and plasma leptin were calculated using the trapezoid rule. This was divided by the observation time period (i.e. 300 min) for a time-averaged value (mmol·L^{-1}).

Whole-body fat and carbohydrate utilization rates were calculated via stoichiometric equations (21), with adjustments made to account for protein utilization and the utilization of muscle glycogen during exercise (21). Within-lab, energy expenditure was determined with lipids, glucose and glycogen giving 9.42, 3.74 and 4.15 $kJ \cdot g^{-1}$ of energy respectively (21). At these exercise intensities, plasma glucose utilization during exercise can be assumed to be equivalent to the plasma glucose rate of disposal (26). Muscle glycogen utilization was calculated as total carbohydrate utilization minus plasma glucose utilization during exercise (this estimate includes the utilization of all non-glucose carbohydrates [e.g. lactate]). To account for excess post-exercise oxygen consumption in BE and FE, estimates of substrate utilization and energy expenditure were adjusted using similar research (27). To assess whether the carbohydrate status of specific tissues (i.e. liver or muscle) were predictors of post-exercise energy intake, plasma glucose and muscle glycogen utilization during exercise in the FE trial, was correlated with compensation in energy intake at lunch (energy intake on FE minus energy intake on BR, normalized to resting metabolic rate [as this measure best reflects the energy requirements of rest]). Only data from the FE trial was included in this analysis because if fasting, plasma glucose utilization during exercise primarily represents the utilization of glucose from hepatic sources but during BE, plasma glucose utilization during exercise reflects liver glucose utilization and glucose from the pre-exercise breakfast.

All data were first tested for normality by using a Shapiro-Wilk test. One-way, repeated measures ANOVAs were used to detect differences between trials at baseline and for summary measures. For multiple comparisons, two-way repeated measures ANOVAs (time x trial) were employed. Degrees of freedom were Greenhouse-Geisser corrected for epsilon <0.75, with Huynh-Feldt corrections used for less severe asphericity. If time x trial interaction effects were identified, multiple paired *t*-tests (with Holm-Bonferroni step-wise adjustments) were used to locate the variance between trials. Pearson *r* were used to explore correlations. Unless otherwise stated, data are means ± normalized (n) 95 % confidence intervals (28). Statistical analyses were performed with commercially

available packages (Graph Pad Prism 7, La Jolla, CA, USA; Microsoft Excel [2013] and IBM SPSS statistics version 22 for windows [IBM, New York, USA]). For three participants, Actiheart^{TM} data did not satisfy the inclusion criteria for useable data and these people were excluded from some analysis.

RESULTS

Energy intake

There was no meaningful compensation through energy intake at lunch, for the energy deficit created by exercise as the mean difference in energy intake in BR *versus* BE was 11 kcal [n95%CI: -182 to 203 kcal] *P*>0.05; [**Figure 2A]**). The omission of breakfast pre-exercise was partially compensated for with lunch energy intake on FE *versus* BE (Figure 2A) but this difference of 166 kcal (n95 % CI: -27 to 308 kcal) was less than the energy in the breakfast (~ 430 kcal). Thus, when accounting for breakfast, within-lab energy intake did not significantly differ in BR and BE, but was significantly higher in BE *versus* FE (265 [123 to 407] kcal; *P*<0.01]. There was some compensation for the energy deficit created by exercise via free-living energy intake in BE, but the omission of breakfast prior to exercise, did not result in any further compensation with energy intake in a free-living environment (Figure 2A). Thus, daily energy intake was significantly higher in BE *versus* BR (245 [81 to 362] kcal; *P*<0.01] and in BE *versus* FE (393 [276 to 510] kcal; *P*<0.01]. The total plasma glucose rate of disposal (Rd) during FE (representing plasma glucose [primarily from hepatic sources] utilization during exercise) was positively correlated with compensation in lunch energy intake in FE *versus* BR when normalized to resting metabolic rate (**Figure 3A**). The total plasma glucose rate of appearance during exercise in FE was also correlated (*r*=0.67, *P*=0.02) with compensation in lunch energy intake (normalized to resting metabolic rate). A similar relationship was apparent when the plasma glucose rate of disposal during FE was correlated with compensation in lunch energy intake when energy intake data were not normalized (*r*=0.61, *P*=0.04) or normalized to DEXA-derived fat free mass (*r*=0.57, *P*=0.05). Muscle glycogen and whole body lipid utilization and energy expenditure during exercise in FE were not correlated with compensation in lunch energy intake (**Figures 3B-D**).

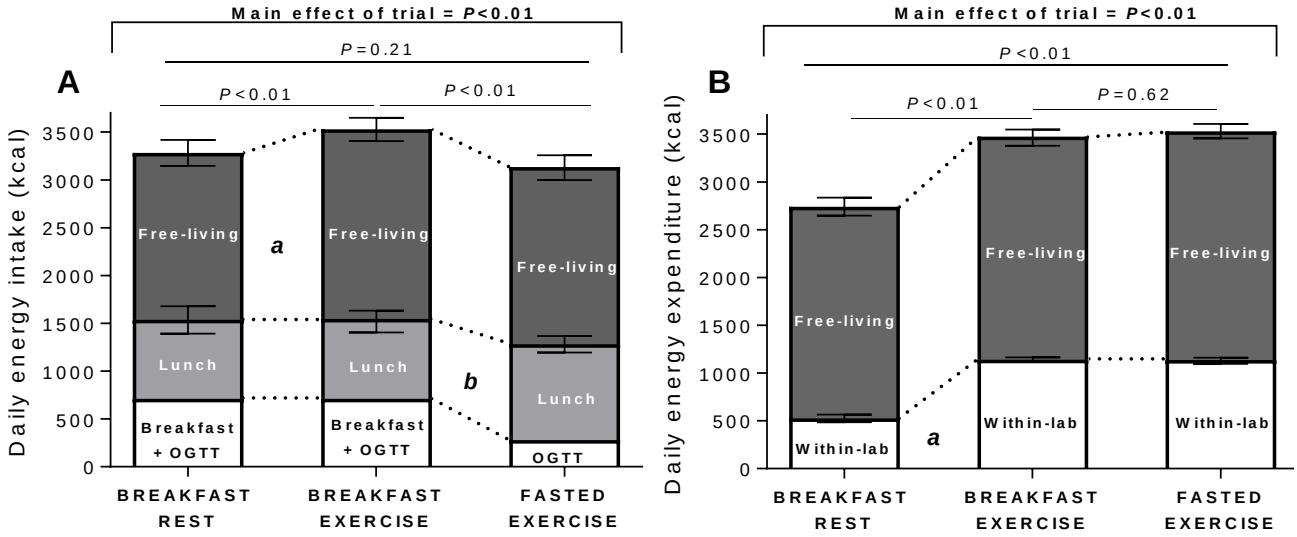

Figure 2. Daily energy intake (**A**) and energy expenditure (**B**). Data are means ± normalized 95 % CI for *n* = 12 (9 for free-living energy expenditure) healthy young men. For (**A**), ***a*** represents a difference in free-living energy intake with breakfast rest and breakfast exercise with *P*<0.05 and ***b*** a difference in lunch energy intake in breakfast exercise *versus* fasted exercise with *P*<0.05. For (**B**) ***a*** shows a difference for within-lab energy expenditure with breakfast rest and both exercise trials with *P*<0.05.

Energy expenditure and substrate utilization

The exercise was completed as prescribed and the intensity was 61 ± 3 % $\dot{V}O_2$ peak (mean ± SD). Energy expenditure during cycling was 683 kcal in BE (n95 % CI: 664 to 702 kcal) and 697 kcal in FE (n95 % CI: 678 to 715 kcal; *P*=0.28 *versus* BE). Carbohydrate utilization was significantly higher in BE (536 [510 to 561] kcal) *versus* FE (478 [452 to 503] kcal, *P*<0.01), but whole-body lipid utilization was significantly lower in BE (138 [102 to 175] kcal) compared to FE (212 [176 to 248] kcal; *P*<0.01). During the OGTT, no difference between the trials in energy expenditure or substrate use was detected. There was no clear difference between any trials for free-living physical activity (**Figure 2B**). Thus, daily energy expenditure was significantly lower with BR *versus* BE and FE, but did not significantly differ in BE *versus* FE (Figure 2B).

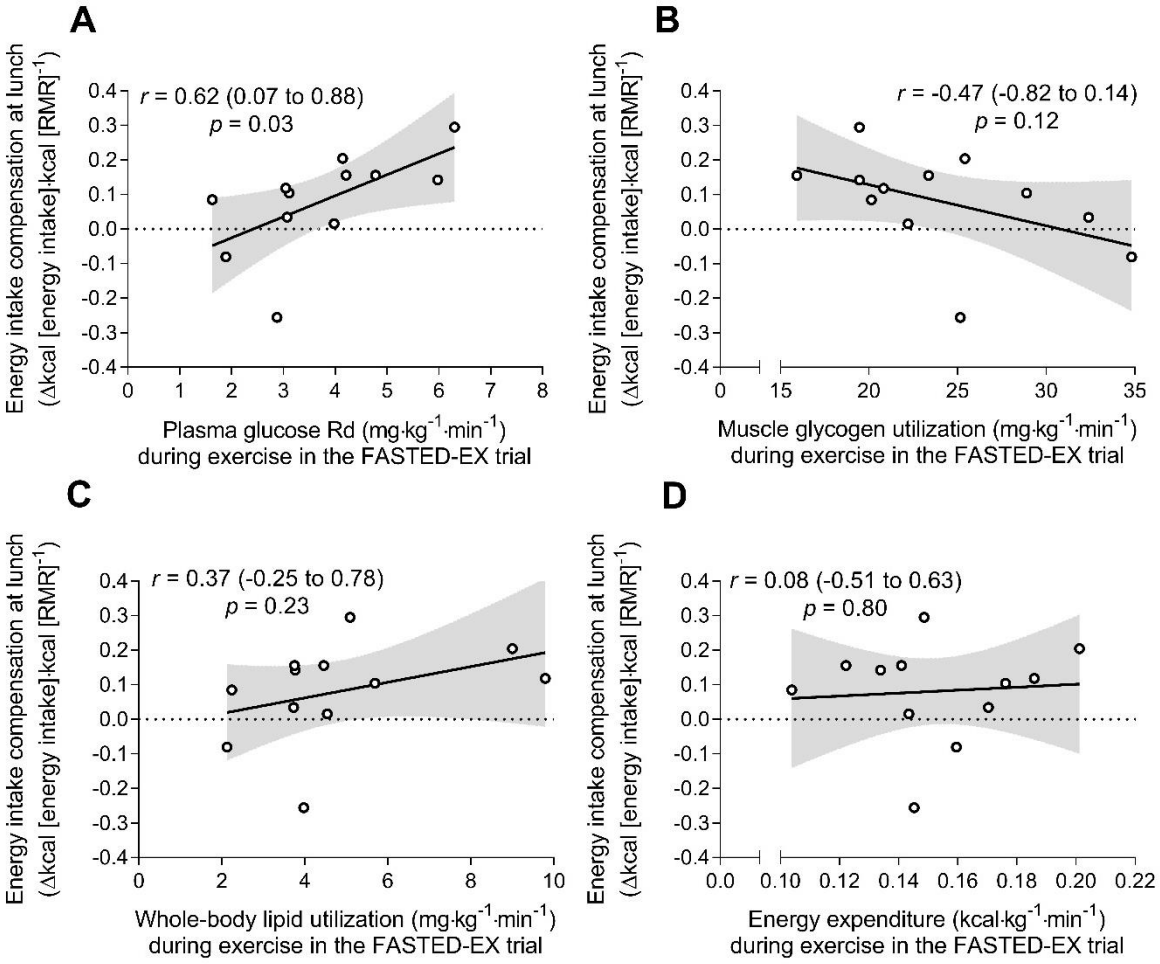

Figure 3. The plasma glucose rate of disappearance (Rd) (**A**), muscle glycogen utilization (**B**), whole-body lipid utilization (**C**) and energy expenditure (**D**) during fasted exercise *versus* lunch energy intake compensation (lunch energy intake after fasted exercise minus lunch energy intake after rest) normalized to resting metabolic rate. Data are Pearson *r*. *n* = 12 healthy young men.

Energy and substrate balance

Consuming breakfast but performing exercise (BE) resulted in a lower within-lab energy balance *versus* rest (BR), which was primarily driven by a difference in carbohydrate balance (**Figure 4A**). The omission of breakfast prior to exercise (FE) resulted in a lower within-lab energy balance compared to BE, primarily via the induction of a significantly lower fat balance, as no significant difference in carbohydrate balance was apparent in FE *versus* BE (Figure 4A). The same pattern between trials was shown over 24 h (**Figure 4B**), where a higher energy balance was observed in BR compared to BE, and in BE compared to FE.

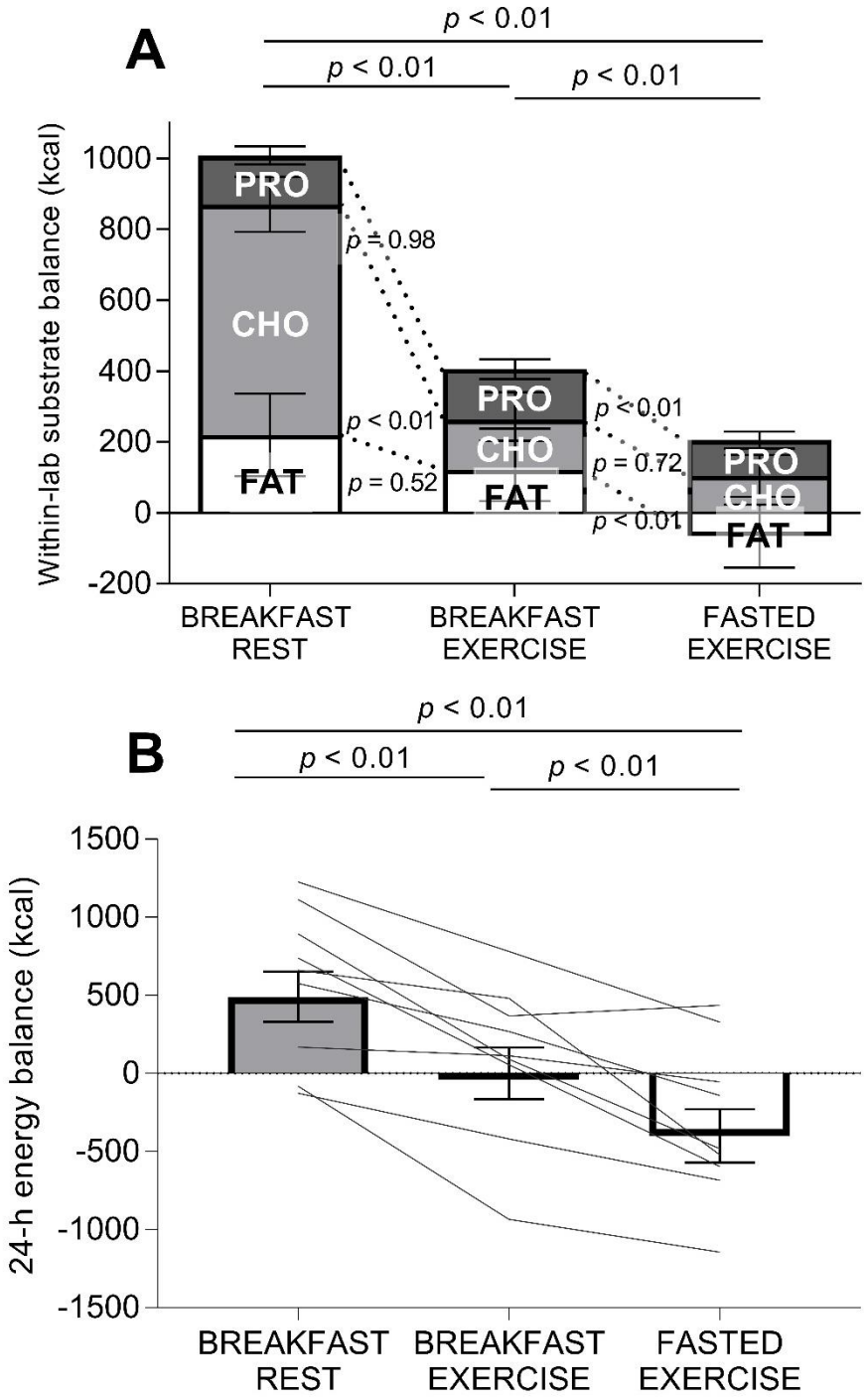

Figure 4. Within-lab (**A**) and daily energy balance (**B**) Data are means ± normalized 95 % CI for *n* = 12 (9 for daily energy balance) healthy young men. PRO = protein; CHO = carbohydrate. For (**A**), ***a*** represents a difference in CHO balance between breakfast rest and breakfast exercise with *P*<0.05 and ***b*** a difference in FAT balance for breakfast exercise and fasted exercise with *P*<0.05.

Plasma leptin and fibroblast growth factor 21 (FGF-21) concentrations

At baseline there were no differences for plasma leptin, nor FGF-21 concentrations (**Figure 5**). A time x trial interaction effect was detected for plasma leptin, but with post-hoc adjustment there was no significant difference at any time point, and no main effect of trial was apparent for the leptin AUC (*P*=0.21). No time x trial interaction effect was observed for FGF-21, and there was no main effect for the FGF-21 AUC (*P*=0.07).

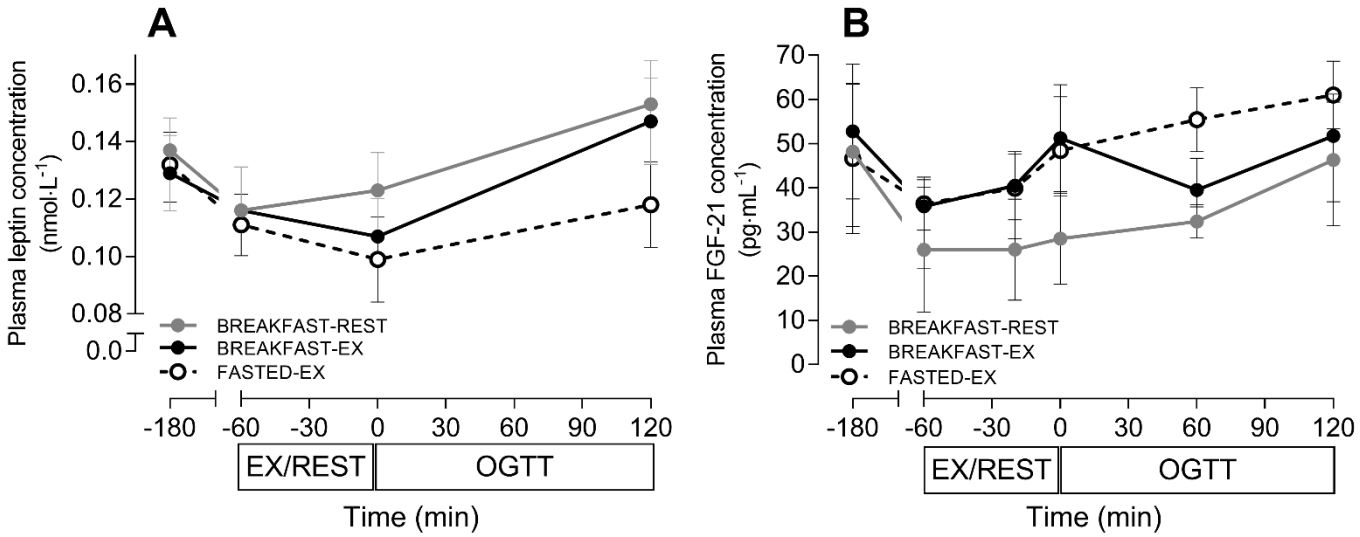

Figure 5. Plasma leptin (**A**) and FGF-21 (**B**) concentrations. Data are means ± normalized 95% CI for *n* = 12 healthy men. OGTT = oral glucose tolerance test.

DISCUSSION

This is the first study to assess the effect of pre-exercise feeding *versus* fasting on all components of energy balance over 24 h, with inclusion of within-lab *and* free-living periods. We showed that breakfast omission before exercise (FE) is not fully compensated for with post-exercise energy intake, and not compensated for at all with subsequent free-living energy expenditure, creating a more negative daily energy balance *versus* breakfast consumption prior to exercise (BE). Our results also demonstrate that plasma glucose utilization during FE demonstrated a stronger relationship with energy intake compensation, than muscle glycogen utilization, whole-body lipid utilization or exercise energy expenditure. Plasma glucose utilization was also the only carbohydrate source to demonstrate a positive relationship with energy intake compensation. As plasma glucose during exercise when fasting is primarily derived from hepatic sources, this result supports a potential role for liver carbohydrate status in the regulation of post-exercise energy balance. These data offer new insights into responses to feeding and exercise that are applicable to typical daily living (29, 30).

We showed that the energy deficit created by exercise was not compensated for through a higher energy intake at an *ad libitum* lunch in an exercise *versus* rest trial (BE *versus* BR), but was partially compensated for with a higher energy intake later in the day (in a free-living setting). In contrast, breakfast omission before exercise (FE) was partially compensated for at lunch, but not further compensated for later in the day. These findings suggest that the compensation for the energy deficits created by exercise and pre-exercise breakfast omission likely occur over different time periods. The energy intake responses we report with pre-exercise breakfast omission are in line with previous observations that when healthy men perform 60-min of running after breakfast, evening and 24-h energy intake are higher *versus* exercise before breakfast (16). As fasting prior to exercise reduces carbohydrate utilization during exercise (13), these results are consistent with a theory that the status of endogenous carbohydrate stores (i.e. liver and muscle glycogen) may play a role in energy balance regulation (7). Further evidence supporting the role of carbohydrate metabolism in regulating

energy balance comes from our current, and prior observations (15) that the more negative within-lab energy balance in the FE trial was attributable to a negative fat balance, as carbohydrate balance was similar to the BE trial.

The ability to fully apply these energy intake findings to daily living is, however, incomplete without an assessment of free-living energy expenditure. Here, we also demonstrate that pre-exercise breakfast ommission is not fully compensated for via non-exercise physical activity energy expenditure, when using objective measures of physical activity in a free-living environment. Thus, we provided a more *complete* picture of energy balance after breakfast *versus* fasting before exercise. Indeed, the physical activity monitor we used has been validated in laboratory and free-living settings (and *versus* doubly-labelled water) (25, 31, 32). Our results show that [1] even with the inclusion of a free-living component, fasting prior to exercise creates a more negative daily energy balance compared to if a pre-exercise breakfast is consumed and that [2] whole-body carbohydrate balance may contribute to energy balance regulation (at least post-exercise).

Previously, it was unclear if a specific carbohydrate store was a primary regulator of post-exercise energy balance. Here, we show that plasma glucose disposal rates and plasma glucose appearance rates during exercise in FE (which primarily represents the mobilization and utilization of glucose from hepatically derived sources, as no carbohydrate was ingested before/during exercise in FE) were the only carbohydrate related outcomes (not muscle glycogen utilization) to demonstrate a positive relationship with energy intake compensation. This is the first evidence of a link between tissue-specific carbohydrate utilization during exercise and energy balance in humans. In our fasting exercise trial, participants with higher rates of plasma glucose disposal consumed more energy at lunch, relative to their rest trial. This finding that a more rapid utilization of plasma glucose during exercise in FE (and a likely higher rate of utilization of carbohydrate from hepatic sources) may increase post-exercise energy intake, is consistent with research showing that a higher liver glycogen content in mice is associated with a lower energy intake and a lower body and fat mass (33). That

result suggests that the liver glycogen content may be a regulator of energy balance, and is consistent with the correlation we report because higher liver glucose utilization rates would be expected to deplete the liver glycogen content during exercise to a greater extent. Future studies should confirm this relationship using a direct measure of net hepatic glycogen utilization, like ^{13}C magnetic resonance spectroscopy.

A possible link between the liver carbohydrate status and post-exercise energy intake regulation may be explained by concentrations of circulating hormones. For example, exercise increases liver FGF-21 secretion (34), which may influence subsequent energy intake (35). It is also suggested that blood leptin concentrations may help to regulate carbohydrate metabolism and energy balance during periods of fasting (36). During rest, higher plasma leptin concentrations after a lunch meal have been previously observed in a breakfast *versus* morning fasting trial (14). This may be accounted for by a slow release of leptin in response to breakfast and/or a diurnal shift in leptin release with morning fasting (14). However, whilst confirming that exercise increases plasma FGF-21 concentrations, we showed that neither plasma FGF-21, nor plasma leptin concentrations, were altered by pre-exercise breakfast omission (FE) *versus* pre-exercise breakfast consumption (BE). The postprandial leptin response we observed *after exercise* was, however, similar to the response observed with breakfast ommission *at rest.* Alternatively, a higher rate of liver carbohydrate utilization may influence energy balance via a liver-brain neural network (the hepatic branch of the vagus nerve) to the central nervous system, as has been demonstrated in rodents (12). Although future research needs to further investigate any mechanisms linking overall and liver carbohydrate metabolism to energy balance in humans, the evidence presented here is the first to demonstrate that plasma glucose utilization during fasted exercise (and thus carbohydrate use from hepatic sources) is positively correlated with post-exercise energy intake in humans. Our results are most applicable to young physically active men. However, as untrained individuals show increased liver glucose utilization during endurance-type exercise (37), it is possible that these people

may be more susceptible to a high post-exercise energy intake, which may make it more difficult for these individuals to lose weight via exercise.

Although the regulatory mechanisms remain unclear, this study provides novel insights into the regulation of *all* behavioural components of daily energy balance after pre-exercise breakfast omission (FE) *versus* breakfast consumption (BE). Our findings must however be interpreted in light of the study design. Firstly, although the protocol included a free-living period, participants were constrained to a laboratory environment during the morning, and it is possible that behaviours that alter energy balance may have changed in a free-living environment during this time after fasting *versus* fed exercise. This restriction of physical activity likely contributed to the positive 24-h energy balance observed in the BR trial. In addition, participants also had a limited choice of food during the study and it is possible that energy intake may have differed under more free-living conditions. Our results also only relate to breakfast *omission* and not altered breakfast *timing*. Despite this, under energy balanced conditions (with the consumption of breakfast either pre- or post-exercise) energy intake at an *ad libitum* lunch does not differ, despite alterations in *substrate* balance (38). In light of that result and our current findings, future research should now investigate all components of energy balance with altered timing of breakfast and in free-living settings (rather than breakfast omission and the time restricted feeding with fasting prior to exercise). Investigating energy balance responses to regular exercise in the fasting *versus* fed state and with overweight populations should be another focus for future work. This would provide insights as to whether the 24-h energy balance responses we report here, translate into enduring changes in body mass or composition with repeated bouts of exercise in the fasting *versus* fed state. Finally, although this study lacks characterization of other gut hormones that have been associated with energy intake, it has been shown that acylated ghrelin and glucagon like peptide 1 (GLP-1) responses to exercise in the fasting *versus* fed states do not differ to any meaningful degree (15, 39, 40). Plasma insulin concentrations during and after exercise were also not altered to any great extent by pre-exercise feeding in our participants (17).

To conclude, pre-exercise breakfast omission is not fully compensated for with energy intake, nor at all with non-exercise energy expenditure post-exercise, creating a more negative 24-h energy balance compared to when breakfast is consumed before exercise. We also show that post-exercise energy intake compensation is positively correlated with plasma glucose utilization when exercise is performed when fasting, highlighting a possible role for liver carbohydrate status in energy balance regulation. These results have important implications for the regulation of post-exercise energy balance and suggest that for healthy young men, a short-term energy deficit may be more easily attained if breakfast is omitted before exercise.

REFERENCES FOR CHAPTER 5

1. Donnelly JE, Blair SN, Jakicic JM, Manore MM, Rankin JW, Smith BK. American College of Sports Medicine Position Stand. Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults. Med Sci Sports Exerc 2009; 41:459-471.
2. King NA, Hopkins M, Caudwell P, Stubbs R, Blundell JE. Individual variability following 12 weeks of supervised exercise: identification and characterization of compensation for exercise-induced weight loss. Int J Obes 2008; 32:177-184.
3. Turner JE, Markovitch D, Betts JA, Thompson D. Nonprescribed physical activity energy expenditure is maintained with structured exercise and implicates a compensatory increase in energy intake. Am J Clin Nutr 2010; 92: 1009-1016.
4. Thompson D, Peacock OJ, Betts J. Substitution and compensation erode the energy deficit from exercise interventions. Med Sci Sports Exerc 2014; 46:423.
5. Betts JA, Richardson JD, Chowdhury EA, Holman GD, Tsintzas K, Thompson D. The causal role of breakfast in energy balance and health: a randomized controlled trial in lean adults. Am J Clin Nutr 2014; 100:539-547.
6. Chowdhury EA, Richardson JD, Holman GD, Tsintzas K, Thompson D, Betts JA. The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults. Am J Clin Nutr 2016; 103:747-756.
7. Flatt JP. Macronutrient composition and food selection. Obes 2001; Res. 9: 256-262.
8. Hopkins M, Jeukendrup A, King NA, Blundell JE. The relationship between substrate metabolism, exercise and appetite control. Sports Med 2011; 41: 507-521.
9. Hall KD, Guo J. Obesity Energetics: Body Weight Regulation and the Effects of Diet Composition. Gastroenterology 2017; 152:1718-1727.
10. Alméras N, Lavallée N, Després J-P, Bouchard C, Tremblay A. Exercise and energy intake: effect of substrate oxidation. Physiol Behav 1995;57:995-1000.
11. Hopkins M, Blundell JE, King NA. Individual variability in compensatory eating following acute exercise in overweight and obese women. Br J Sports Med (Epub; DOI: 10.1136/bjsports-2012-091721).
12. López-Soldado I, Fuentes-Romero R, Duran J, Guinovart JJ. Effects of hepatic glycogen on food intake and glucose homeostasis are mediated by the vagus nerve in mice. Diabetologia 2017;60:1076-83.
13. Enevoldsen L, Simonsen L, Macdonald I, Bülow J. The combined effects of exercise and food intake on adipose tissue and splanchnic metabolism. The Journal of Physiology 2004; 561: 871-882.
14. Chowdhury EA, Richardson JD, Tsintzas K, Thompson D, Betts JA. Carbohydrate-rich breakfast attenuates glycaemic, insulinaemic and ghrelin response to ad libitum lunch relative to morning fasting in lean adults. Br J Nutr 2015; 114: 98-107.
15. Gonzalez JT, Veasey RC, Rumbold PL, Stevenson EJ. Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. Br J Nutr 2013;110:721-32.
16. Bachman JL, Deitrick RW, Hillman AR. Exercising in the Fasted State Reduced 24-Hour Energy Intake in Active Male Adults. J Nutr Metab. 2016; (Epub; DOI: 10.1155/2016/1984198)
17. Edinburgh RM, Hengist A, Smith HA, Travers RL, Koumanov F, Betts JA, Thompson D, Walhin J-P, Wallis GA, Hamilton DL et al. Pre-Exercise Breakfast Ingestion versus Extended Overnight Fasting Increases Postprandial Glucose Flux after Exercise in Healthy Men. Am J Physiol Endocrinol Metab 2018; (Epub ahead of print; DOI: 10.1155/2016/1984198)

18. Deighton K, Frampton J, Gonzalez J. Test-meal palatability is associated with overconsumption but better represents preceding changes in appetite in non-obese males. Br J Nutr 2016;116:935-43.
19. Radziuk J. An integral equation approach to measuring turnover in nonsteady compartmental and distributed systems. Bull Math Biol 1976; 38: 679-693.
20. Radziuk J, Norwich KH, Vranic M. Experimental validation of measurements of glucose turnover in nonsteady state. Am J Physiol Endocrinol Metab 1978; 234:E84.
21. Jeukendrup A, Wallis G. Measurement of substrate oxidation during exercise by means of gas exchange measurements. Int J Sports Med 2005;26:28-37.
22. Brage S, Brage N, Franks P, Ekelund U, Wareham N. Reliability and validity of the combined heart rate and movement sensor Actiheart. Eur J Clin Nutr 2005;59:561-70.
23. Brage S, Brage N, Franks PW, Ekelund U, Wong M-Y, Andersen LB, Froberg K, Wareham NJ. Branched equation modeling of simultaneous accelerometry and heart rate monitoring improves estimate of directly measured physical activity energy expenditure. J. Appl. Physiol. 2004;96:343-51.
24. Brage S, Ekelund U, Brage N, Hennings MA, Froberg K, Franks PW, Wareham NJ. Hierarchy of individual calibration levels for heart rate and accelerometry to measure physical activity. J. Appl. Physiol. 2007;103:682-92.
25. Brage S, Westgate K, Franks PW, Stegle O, Wright A, Ekelund U, Wareham NJ. Estimation of free-living energy expenditure by heart rate and movement sensing: a doubly-labelled water study. PloS one 2015; (Epub; DOI: 10.1371/journal.pone.0137206).
26. Jeukendrup AE, Wagenmakers AJM, Stegen JHCH, Gijsen AP, Brouns F, Saris WHM. Carbohydrate ingestion can completely suppress endogenous glucose production during exercise. Am J Physiol Endocrinol Metab 1999;276:672-83.
27. Edinburgh R, Hengist A, Smith HA, Betts JA, Thompson D, Walhin J-P, Gonzalez JT. Prior exercise alters the difference between arterialised and venous glycaemia: implications for blood sampling procedures. Br J Nutr 2017; 10: 1414-1421.
28. Loftus GR, Masson MEJ. Using confidence intervals in within-subject designs. Psychon Bull Rev 1994; 1:476-90.
29. de Castro JM. The time of day of food intake influences overall intake in humans. J Nutr 2004;134:104-11.
30. Ruge T, Hodson L, Cheeseman J, Dennis AL, Fielding BA, Humphreys SM, Frayn KN, Karpe F. Fasted to fed trafficking of fatty acids in human adipose tissue reveals a novel regulatory step for enhanced fat storage. J Clin Endocrinol Metab 2009;94:1781-8.
31. Villars C, Bergouignan A, Dugas J, Antoun E, Schoeller DA, Roth H, Maingon A-C, Lefai E, Blanc S, Simon C. Validity of combining heart rate and uniaxial acceleration to measure free-living physical activity energy expenditure in young men. J. Appl. Physiol 2012;113:1763-71.
32. Thompson D, Batterham AM, Bock S, Robson C, Stokes K. Assessment of low-to-moderate intensity physical activity thermogenesis in young adults using synchronized heart rate and accelerometry with branched-equation modeling. J Nutr 2006;136:1037-42.
33. López-Soldado I, Zafra D, Duran J, Adrover A, Calbó J, Guinovart JJ. Liver Glycogen Reduces Food Intake and Attenuates Obesity in a High-Fat Diet–Fed Mouse Model. Diabetes 2015;64:796-807.
34. Kim KH, Kim SH, Min YK, Yang HM, Lee JB, Lee MS. Acute exercise induces FGF21 expression in mice and in healthy humans. PLoS One 2013; (Epub; DOI: 10.1371/journal.pone.0063517).
35. von Holstein-Rathlou S, BonDurant LD, Peltekian L, Naber MC, Yin TC, Claflin KE, Urizar AI, Madsen AN, Ratner C, Holst B et al. FGF21 Mediates Endocrine Control

of Simple Sugar Intake and Sweet Taste Preference by the Liver. Cell Metab 2016;23:335-43.
36. Perry RJ, Wang Y, Cline GW, Rabin-Court A, Song JD, Dufour S, Zhang XM, Petersen KF, Shulman GI. Leptin Mediates a Glucose-Fatty Acid Cycle to Maintain Glucose Homeostasis in Starvation. Cell 2018; 234-248.
37. Gonzalez J, Betts J. Dietary Sugars, Exercise and Hepatic Carbohydrate Metabolism: Sugars and liver metabolism. Proc Nutr Soc 2018; (Epub ahead of print; DOI: 10.1017/S0029665118002604).
38. Farah NM, Gill JM. Effects of exercise before or after meal ingestion on fat balance and postprandial metabolism in overweight men. Br J Nutr 2013;109:2297-307.
39. Clayton DJ, Barutcu A, Machin C, Stensel DJ, James LJ. Effect of breakfast omission on energy intake and evening exercise performance. Med Sci Sports Exerc 2015;47:2645-52.
40. Scott JP, Sale C, Greeves JP, Casey A, Dutton J, Fraser WD. Effect of fasting versus feeding on the bone metabolic response to running. Bone. 2012;51:990-9.

CHAPTER 6 - BREAKFAST-EXERCISE TRAINING STUDY

Lipid metabolism links nutrient-exercise timing to insulin sensitivity in overweight men.

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Acknowledgements:

The authors thank Russell Davies, Esther Punter, Emily Fallon, Josh Dominy and Lauren Davey for assisting and/or supervising some of the training sessions, Marine Camus for advice on Western blotting and Laura Wood for assisting with Western blotting. We also thank all those who participated in the studies.

Sources of Support:

This study was supported by The Physiological Society (UK) and the Allen Foundation Inc. (USA), N-FA was supported by a PhD Scholarship from the Ministry of Education, Malaysia. FK is funded by the Medical Research Council (MR/P002927/1) and FMB by the Medical Research Council (MR/S008144/1).

Trial Registrations: https://clinicaltrials.gov/ [NCT02744183; NCT02397304]

Data Access Statement: The complete data set relating to this manuscript can be found at: https://doi.org/10.15125/BATH-00672.

RELEVANCE TO THESIS

Nutrient availability before, during and after exercise potently alters metabolism during- and in the hours after-exercise (10, 15). Specifically, exercise performed in an overnight fasted-state increases lipid utilisation and also alters the activity of proteins implicated in exercise adaptation for a number of hours post-exercise, *versus* exercise after consuming carbohydrates. This raises the possibility that nutrient-exercise timing could regulate health-related adaptions to exercise via adaptations in skeletal muscle and/or altering lipid flux. Indeed, as discussed in **Chapter 1**, a higher turnover of lipids derived from the adipose tissue has been associated with a better regulation of metabolic health (167, 168) and a regular utilisation of lipid stores in skeletal muscle is associated with increased peripheral insulin sensitivity, potentially by alleviating an accumulation of lipid intermediates that can interfere with intramuscular insulin signalling (9).

Whilst characterising acute metabolic and intramuscular responses to exercise can provide insights into the potential utility of exercise-nutrient interactions to improve health outcomes, the acute responses to exercise and/or nutrition do not consistently translate into the expected chronic adaptations with regular training. One example is that exercise performed in a fasted state *versus* after consuming carbohydrates can increase the expression or activity of proteins involved in lipid metabolism and also increases the capacity for utilising lipids during exercise at the whole-body level with repeated training (305, 306). These adaptations should, in theory, improve exercise capacity or performance in endurance-type events by reducing the reliance on endogenous carbohydrate stores. However, in most of these studies, no clear differences are shown between exercise groups for the changes in endurance-type exercise performance after training. As such, even if a higher rate of lipid utilisation when breakfast is omitted *versus* consumed before an acute bout of exercise creates the theoretical conditions for improvements in insulin sensitivity, this needs to be confirmed with exercise training studies. The fact that acute responses to exercise and/or nutrient ingestion do not consistently translate into the expected enduring adaptations with repeated exposure to these stimuli is not the only reason why training studies are now required in this area.

Indeed, there is currently a lack of exercise training studies investigating nutrient-exercise interactions in a context of metabolism and health. This is likely to be important, because in the absence of marked weight loss, some exercise training studies report a modest group-level effect for insulin sensitivity and also apparent inter-individual variability in this regard (488, 489). This may be explained by differences in the nutritional status of participants before exercise.

If healthy men consume a hypercaloric (+ 30 % kcal), fat-rich diet (50 % kcal), whole-body glucose tolerance and oral-glucose tolerance test derived estimates of peripheral insulin sensitivity are increased over 6 weeks *versus* a non-exercise control group, if exercise is performed before breakfast, but not if carbohydrates are consumed before/during exercise (11). This suggests that nutrient-exercise timing mediates health-related adaptations to exercise. However, a high-fat diet is not representative of the diet consumed by many people living in developed countries and in that study, changes in oral-glucose insulin sensitivity may have been due to increases in body mass in the control and fed-exercise groups, but not in the fasted-exercise group. Nonetheless, that study, and other data from lean, healthy men suggests that nutrient provision prior to and/or during exercise alters adaptive responses to moderate-intensity exercise training (490). However, humans classified as overweight or obese can display divergent metabolic responses to feeding and fasting compared to lean humans (491). Therefore, in order to understand the potential for nutrient-exercise timing to alter metabolic health in individuals at risk of metabolic diseases, there is a need to study relevant populations, such as people classified as overweight. Thus, the aim of the research in **Chapter 6** was to determine the effects of endurance-type exercise training before *versus* after nutrient-intake on oral glucose insulin sensitivity in men classified as overweight and to explore potential underlying mechanisms.

This declaration concerns the article entitled:			
Lipid metabolism links nutrient-exercise timing to insulin sensitivity in overweight men.			
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [X] **Accepted** [ ] **Published** [ ]			
Publication details (reference)	Edinburgh, R.M., Bradley, H.E., Abdullah, N-F., Robinson, S.L., Chrzanowski-Smith, O., Walhin, J-P., Joanisse S., Manolopoulos, K., Philp, A., Hengist, A., Chabowski, A., Brodsky, F.M., Koumanov, F., Betts, J.A., Thompson, D., Wallis, G. A., Gonzalez, J.T. (2019).		
Copyright status (tick the appropriate statement) I hold the copyright for this material [ ] Copyright is retained by the publisher, but I have been given permission to replicate the material here [X]			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	This project had two experiments (an **acute study** and a **training study**). **Formulation of ideas:** RME and JTG formulated the ideas for the **training study** and GAW formulated the ideas for the **acute study**. **Design of methodology:** RME and JTG predominantly designed the methodology for the **training study** and GAW designed the methodology for the **acute study**. **Experimental work:** RME (> 95 %), JTG, OCS AH, and J-PW conducted the research for the **training study**, RME analyzed all of the samples for the **training study** (blood and muscle analysis [with the help of FK] except for the phospholipid content [analysed by AC]), RME was present for all data collection on trial days, prepared and analysed the samples, completed statistical analysis and most aspects of the experimental work for the **training study**. The **acute study** was completed at the University of Birmingham. **Presentation of data in journal format:** RME (> 80 %) and JTG primarily wrote the paper, and all authors contributed to earlier versions of the manuscript and approved the final version of the manuscript.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	R.M.Edinburgh	**Date**	2nd July 2019

5799

Abstract

Context: Pre-exercise nutrient availability alters acute metabolic responses to exercise, which could modulate training responsiveness. We hypothesised that in men with overweight/obesity, acute exercise before *versus* after nutrient ingestion would increase whole-body and intramuscular lipid utilization, translating into greater increases in oral glucose insulin sensitivity over 6-weeks of training.

Design and Participants: We showed in men with overweight/obesity (mean±SD for BMI: 30.2±3.5 $kg \cdot m^{-2}$ for acute, crossover study, 30.9±4.5 $kg \cdot m^{-2}$ for randomized, controlled, training study) a single exercise bout before *versus* after nutrient provision increased lipid utilisation at the whole-body level, but also in both type I ($p<0.01$) and type II muscle fibres ($p=0.02$). We then used a 6-week training intervention to show sustained, 2-fold increases in lipid utilisation with exercise before *versus* after nutrient provision ($p<0.01$).

Main Outcome Measures: Postprandial glycemia was not differentially affected by exercise training before *vs* after nutrient provision ($p>0.05$), yet plasma insulinemia was reduced with exercise training before, but not after nutrient provision ($p=0.03$), resulting in increased oral glucose insulin sensitivity when training was performed before *versus* after nutrient provision (25±38 *vs* -21±32 $mL \cdot min^{-1} \cdot m^{-2}$; $p=0.01$) and this was associated with increased lipid utilisation during exercise ($r=0.50$, $p=0.02$). Regular exercise prior to nutrient provision augmented remodelling of skeletal muscle phospholipids and protein content of the glucose transport protein GLUT4 ($p<0.05$).

Conclusions: Experiments investigating exercise training and metabolic health should consider nutrient-exercise timing, and exercise performed before *versus* after nutrient intake (i.e., in the fasted state) may exert beneficial effects on lipid utilisation and reduce postprandial insulinemia.

INTRODUCTION

Postprandial hyperinsulinemia and associated peripheral insulin resistance are key drivers of metabolic diseases, such as type 2 diabetes (T2D) and cardiovascular disease (1-3). Obesity and a sedentary lifestyle are independently associated with changes in skeletal muscle that can reduce insulin sensitivity (4,5) and increase hyperinsulinemia, contributing to elevated cardiovascular disease risk (2). Therefore, increasing insulin sensitivity and reducing postprandial insulinemia are important targets for interventions to reduce the risk of metabolic disease.

Regular exercise training represents a potent strategy to increase peripheral insulin sensitivity and to reduce postprandial insulinemia (6). The beneficial effects of exercise on oral glucose tolerance and insulin sensitivity can be attributed to both an 'acute phase' (during and straight after each bout of exercise performed) and the more enduring molecular adaptations that accrue in response to regular exercise (7). A single bout of endurance-type exercise activates contractile pathways in exercising muscle, which (independently of insulin) translocate the glucose transporter, GLUT4, to the plasma membrane and T-tubules to facilitate increased transmembrane glucose transport (8-10). The mechanisms that underlie the exercise-training induced increases in oral glucose insulin sensitivity include an increase in the total amount of time spent in the 'acute phase' (7), but also other adaptations that occur, such as changes in body composition (e.g. increased fat-free mass and reduced adiposity), an increased mitochondrial oxidative capacity (11), adaptations relating to glucose transport and insulin signaling pathways (12), and alterations to the lipid composition of skeletal muscle (13,14).

Despite the potential for exercise to increase whole-body and peripheral insulin sensitivity, there can be substantial variability in the insulin sensitizing effects of fully-supervised exercise training programs (15). Crucially, this inter-individual variability for postprandial insulinemia following exercise training has also been shown to be greater than that of a control group (15), which demonstrates that

some of this variability to exercise is true inter-individual variability (16). Nutritional status and thus the availability of metabolic substrates alters metabolism during and following exercise (17-20). Carbohydrate feeding before and during exercise suppresses whole-body and skeletal muscle lipid utilization (21,22) and blunts the skeletal muscle mRNA expression of genes involved in exercise-adaptation for many hours post-exercise (23-25). This raises the possibility that nutrient-exercise interactions may regulate adaptive responses to exercise and thereby contribute to the apparent individual variability in exercise responsiveness *via* skeletal muscle adaptation and/or pathways relating to substrate metabolism.

Emerging data in lean, healthy men suggests that nutrient provision affects adaptive responses to exercise training (26,27). However, feeding and fasting may exert different physiological responses in people who are overweight or obese compared to lean individuals. For example, extended morning fasting *versus* daily breakfast consumption upregulates the expression of genes involved in lipid turnover in adipose tissue in lean, but not in obese humans (28). Therefore, in order to fully understand the potential for nutrient-exercise timings to alter metabolism, exercise-adaptations and metabolic health in individuals at increased risk of metabolic disease, there is a need to study the most relevant populations, such as individuals classified as overweight or obese (29). It is currently unknown whether nutrient provision before *versus* after exercise affects adaptations to exercise training in these populations.

To this end, the aim of the present work was to assess the acute and chronic effects of manipulating nutrient-exercise timing on lipid metabolism, skeletal muscle adaptations, and oral glucose insulin sensitivity in men with overweight or obesity. We hypothesized that nutrient-exercise interactions would affect the acute metabolic responses to exercise, with increased whole-body and intramuscular lipid utilization with exercise before *versus* after nutrient provision. We also hypothesized that these acute responses to exercise before *versus* after

nutrient provision would result in greater training-induced increases in oral glucose insulin sensitivity in men classified as overweight or obese.

METHODS

Ethical Approval

This project comprised two experiments. We first assessed the acute metabolic and mRNA responses to manipulating nutrient-exercise timing (**Acute Study**), followed by a 6-week randomized controlled trial to assess the longer-term (i.e. training) adaptations in response to nutrient-exercise timing (**Training Study**). All participants provided informed written consent prior to participation. Potential participants were excluded if they had any condition, or were taking any medication, known to alter any of the outcome measures. The studies were registered at https://clinicaltrials.gov (NCT02397304 and NCT02744183, respectively). Protocols were approved by the National Health Service Research Ethics Committee (15/WM/0128 & 16/SW/0260, respectively) and experiments were conducted in accordance with the Declaration of Helsinki.

Acute Study

In the **Acute Study**, 12 sedentary, men classified as overweight or obese were recruited from the Birmingham region of the UK. The main exclusion criteria included being regularly physically active, having hypertension or possible (undiagnosed) T2D. Participant characteristics are shown in **Table 1**.

This was a randomised cross-over study where on one visit (breakfast-exercise or BR-EX), a standardised breakfast (cornflake cereal with skimmed milk, wholemeal toast, sunflower spread and strawberry jam) was consumed upon arrival at the laboratory (and following 48 h of diet control). The breakfast provided 25% of estimated daily energy requirements [calculated as resting metabolic rate (RMR) multiplied by a physical activity factor of 1.53 (30)] and was 65% carbohydrate, 20% fat and 15% protein. After a 90-min period of rest, 60 min of cycling exercise was then performed at 65% peak oxygen uptake ($\dot{V}O_2$ peak). Expired gas samples were collected at 25-30 min and 55-60 min of exercise to

determine whole-body substrate utilisation rates. Blood was sampled in the overnight-fasted state, at 45 min post breakfast and immediately before exercise was performed (90 min post breakfast), every 30 min during exercise and at 60 min intervals during a 3-h post-exercise recovery. In a subset of participants (*n*=8) *vastus lateralis* muscle was sampled pre- and immediately post-exercise to assess fiber-type specific intramuscular triglyceride (IMTG) and mixed-muscle glycogen utilization. A third muscle sample (taken at 3 h post-exercise) was used to assess the intramuscular gene expression (mRNA) responses to exercise (*n*=7). On the other visit (exercise-breakfast or EX-BR) the participants completed the same protocol, but the breakfast was consumed immediately after the post-exercise muscle sample. The primary outcome for the **Acute Study** was intramuscular lipid utilisation during exercise performed before *versus* after nutrient ingestion.

Training Study

To assess longer-term adaptive (i.e. training) responses to altering nutrient-exercise timing (**Training Study**) we recruited 30, overweight and obese, sedentary men (self-reported non-exercisers) from the Bath region of the UK (**Table 1**). This was a single-blind, randomized, controlled trial, with participants allocated to a no-exercise control group (CON; *n*=9) a breakfast before exercise group (BR-EX; *n*=12) or an exercise before breakfast group (EX-BR; *n*=9) for 6-weeks (**Figure 1**). The exercise was supervised moderate-intensity cycling (Monark Exercise AB, Vansbro, Sweden) performed 3 times per week, starting at 50% peak power output [PPO] (weeks 1-3) and increasing to 55% PPO (weeks 4-6). The duration of the exercise sessions progressed from 30- (week 1) to 40- (week 2) to 50-min (weeks 3-6). All sessions were supervised at the University of Bath. During every one of the 336 exercise training sessions, 1-min expired gas samples were collected every 10 min to assess substrate utilization and heart rate (Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (31) were recorded.

5952

Table 1. Participant characteristics

	Study 1	Study 2 - Training Study			
	Acute Study	**CON**	**BR-EX**	**EX-BR**	***p*-value**
n	*12*	*9*	*12*	*9*	
Body Mass (kg)	95.1 (13.6)	101.1 (19.5)	95.2 (12.4)	98.0 (18.8)	*0.73*
BMI ($kg \cdot m^{-2}$)	30.2 (3.5)	31.8 (5.8)	30.3 (3.9)	30.8 (4.1)	*0.75*
Waist Circumference (cm)	105.7 (11.6)	107.7 (14.8)	103.9 (8.9)	104.7 (11.6)	*0.63*
Hip Circumference (cm)	110.9 (6.5)	110.8 (8.4)	111.4 (7.1)	111.6 (8.5)	*0.32*
Waist-to-Hip Ratio	0.95 (0.08)	0.97 (0.06)	0.93 (0.04)	0.94 (0.05)	*0.31*
$\dot{V}O_2$peak ($ml \cdot kg^{-1} \cdot min^{-1}$)	29.1 (5.3)	32.6 (7.7)	34.3 (5.6)	32.4 (4.0)	*0.71*
PPO (W)	156 (39)	204 (47)	208 (26)	203 (22)	*0.73*
Physical Activity Level (PAL)	-	1.71 (0.16)	1.68 (0.16)	1.68 (0.11)	*0.90*

Data are means (SD) for overweight or obese men. BMI = Body Mass Index; $\dot{V}O_2$peak = peak oxygen uptake; PPO = peak power output; CON = control; BR-EX = breakfast-exercise; EX-BR = exercise-breakfast. Physical Activity Level (PAL) was total daily energy expenditure divided by resting metabolic rate (RMR).

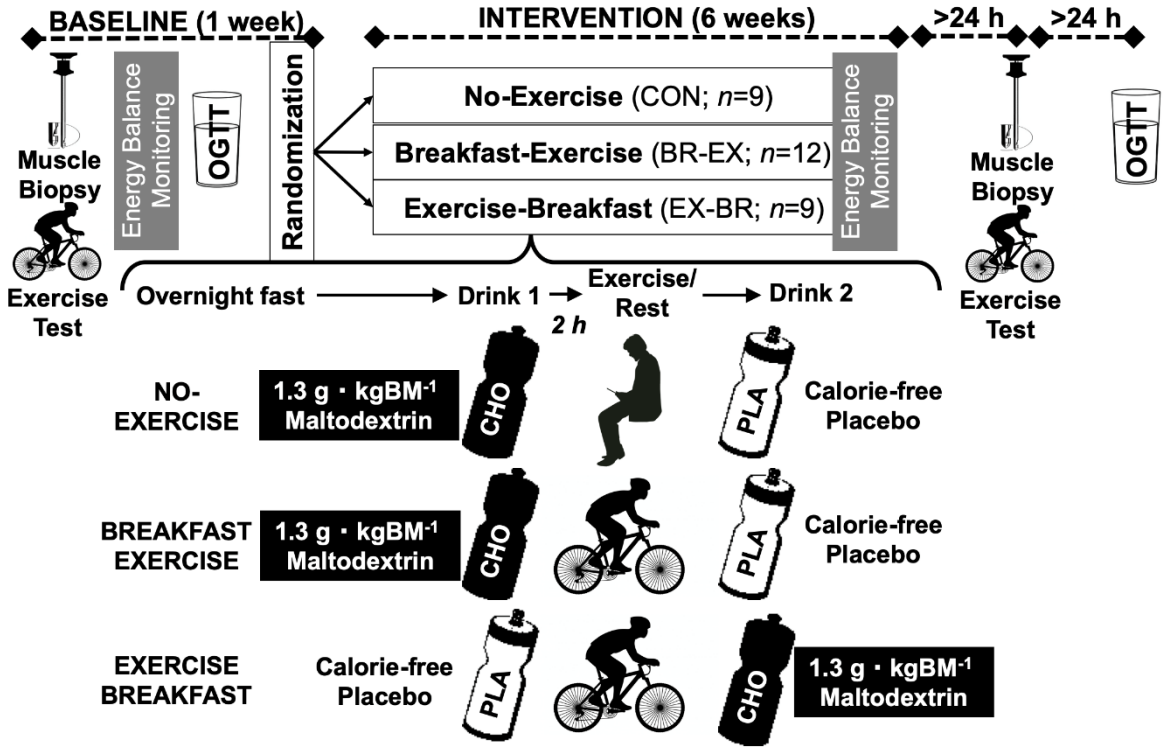


Figure 1. Protocol schematic for the training study.

Participants ate their evening meal before 2000 h the evening prior to any exercise sessions. Participants in BR-EX were given a drink in an opaque bottle made from 1.3 g carbohydrate·kg body mass^{-1} maltodextrin (MyProtein, Northwich, UK) with vanilla flavoring (20% carbohydrate solution) for consumption 2-h before exercise. They were asked not to eat or drink anything else (except water *ad libitum*) in this period and confirmed they had consumed the drink before exercising. After exercise, they were provided a taste matched placebo (water and vanilla flavoring) to consume 2-h after exercise and were asked not to consume anything else during this period. Participants in EX-BR were given the same drinks, but with the order of the drinks reversed. Participants in CON were given the same drinks for three days per week during the intervention, with the carbohydrate drink as breakfast (0800-0900 h) and the placebo for consumption with their lunch (1100-1300 h). These participants were asked not to consume anything else between the drinks. There were no other diet controls in the intervention. Blinding of the groups was deemed successful, because at exit interview, 25 participants (83%) revealed they could not detect a

difference between the carbohydrate and placebo drinks or could not identify which contained carbohydrate. Five participants determined which drink had carbohydrate (CON *n*=1, BR-EX *n*=2, EX-BR *n*=1), but this is within the proportion that could do so at random.

Pre- and post-intervention, an oral glucose tolerance test (OGTT), a *vastus lateralis* muscle sample (fasting, rested state) and an exercise test (to assess $\dot{V}O_2$ peak and the capacity for lipid utilization during exercise in the fasted-state) were undertaken. Post-intervention tests were between 24 h to 48 h (for muscle sampling) and 48 h to 72 h (for OGTT) after the last exercise training session, to reduce any residual effects of the last exercise bout performed on these measurements. The primary outcome for the **Training Study** was the pre- to post-intervention change in the glycaemic and insulinemic responses to the OGTT, which were also used to derive an index of oral glucose insulin sensitivity (as described subsequently).

Pre-trial standardizations

For both studies, the participants were asked to maintain their normal physical activity behaviors and to abstain from alcoholic and caffeinated drinks for 24 h prior to all main laboratory trials. Food intake ceased at 2000 h ± 1 h on the evening before testing and participants fasted overnight (minimum of 10 h). For all trials, participants arrived at the laboratory at 0800 ± 1 h, with the exact time replicated for subsequent trials. For the **Acute Study**, participants were provided with a standardized weight maintaining diet (50% carbohydrate, 35% fat, 15% protein) based on their estimated energy requirements (RMR multiplied by the physical activity factor of 1.53 as stated previously) for consumption for 48 h prior to main trials. For the **Training Study**, they recorded the composition of their evening meal on the day before a pre-intervention trial and replicated this meal for the post-intervention trial, in line with guidelines for testing postprandial glycemic control (32). We have shown that this protocol produces fasting muscle and liver glycogen and fasting intramuscular lipid concentrations that are consistent across trial days (33).

Anthropometry

Stature was measured to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK). Body mass was measured to the nearest 0.1 kg using electronic weighing scales (**Acute Study**: OHaus Champ II Scales, USA; **Training Study**: BC543 Monitor, Tanita, Japan). Waist and hip circumferences were measured to the nearest 0.1 cm and according to the World Health Organization guidelines.

Exercise tests

Participants completed exercise tests on an electronically-braked ergometer. In the **Acute Study**, the starting intensity was 35 W, and this was increased by 35 W every 3 min until volitional exhaustion. In the **Training Study**, the starting intensity for the exercise test was 50 W which was increased by 25 W every 3 min. Heart rate (Polar Electro Oy, Kempele, Finland) and continuous breath-by-breath measurements were recorded (**Acute Study**: Oxycon Pro, Jaeger, Wurzburg, Germany; **Training Study**: TrueOne2400, ParvoMedics, Sandy, USA). Volume and gas analyzers were calibrated using a 3-L calibration syringe (Hans Rudolph, Kansas City, USA) and a calibration gas (16.04% O_2, 5.06% CO_2; BOC Industrial Gases, Linde AG, Germany). Peak power output (PPO) was calculated as the work rate of the final completed stage, plus the fraction of time in the final non-completed stage, multiplied by the W increment. $\dot{V}O_2$ peak was the highest measured $\dot{V}O_2$ over a 30 s period, using methods and attainment criteria previously reported (34).

Blood sampling and analysis

In the **Acute Study**, 10 mL blood was sampled from an antecubital forearm vein and 6 mL was dispensed into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) and centrifuged (4°C at 3500 rpm) for 15 min (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Resultant plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before longer-term storage at -80°C. A proportion of the sample (4 mL) was allowed to clot in a plain vacutainer prior to centrifugation, for serum. Samples were analyzed for plasma glucose, glycerol

and NEFA using an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK). Serum insulin concentrations were measured with an ELISA kit (Invitrogen; Cat#KAQ1251) and Biotek ELx800 analyzer (Biotek Instruments, Vermont, USA).

In the **Training Study**, prior to blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest, a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample in the overnight-fasted state (35). Then a 75-g OGTT was completed and arterialized blood sampled every 15 min for 2 h and processed (as detailed above) for plasma. Plasma glucose (intra-assay CV: 2.50%), glycerol, triglyceride (glycerol-blanked), and total- HDL- and LDL-cholesterol concentrations were measured using an automated analyzer (Daytona; Randox Lab, Crumlin, UK). Plasma insulin (Mercodia AB; reference #10-1113-01) and C-peptide (Sigma Aldrich; reference #EZHCP-20K) concentrations were measured using commercially available ELISA kits (intra-assay CV for insulin: 3.86% and for C-peptide: 4.26%). Non-esterified fatty acid (NEFA) concentrations were assessed via an enzymatic colorimetric kit (WAKO Diagnostics; references #999-34691/#991-34891; intra-assay CV: 7.95%). All analysis was done in batch and for a given participant all samples were included on the same plate.

Muscle sampling

All *vastus lateralis* skeletal muscle samples were collected under local anesthesia (~ 5 mL 1% lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK) and from a 3-6-mm incision at the anterior aspect of the thigh using a 5-mm Bergstrom biopsy needle technique adapted for suction. For the **Acute Study**, samples were collected pre- and immediately post-exercise and at 3 h post-exercise. To enable the analysis of the IMTG content ~ 15-20 mg of each sample was embedded in Tissue-Tek OCT (Sigma Aldrich, Dorset, UK) on cork disc and frozen in liquid nitrogen cooled isopentane, before being transferred into an aluminium cryotube and stored at -80°C. Remaining muscle (for glycogen and gene expression analysis) was frozen in liquid nitrogen and stored at -80°C. In the **Training Study**,

samples were collected pre- and post-intervention with participants in a fasted, resting state, with both samples collected from their dominant leg. Muscle was extracted from the needle and frozen in liquid nitrogen, before storage at -80 °C. Frozen wet muscle (80-100 mg) was freeze-dried and powdered, with visible blood and connective tissue removed. Ice cold lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Sodium deoxycholate; 0.1% SDS and 0.1% NP-40) with protease and phosphatase inhibitors was added. Samples were homogenized with a dounce homogenizer, before a 60 min incubation (4°C with rotation) and 10 min centrifugation (4°C and 20,000 g). The protein content of the resultant supernatant was measured using a bicinchoninic acid assay.

Intramuscular triglyceride (Acute Study)

The muscle mounted in Tissue-Tek was cut into 5 µm thick transverse sections with a cryostat at -25°C (Bright 5040, Bright Instrument Company; Huntingdon, England) and collected onto an uncoated glass slide and frozen immediately after sectioning. Each slide had 4 samples for a participant (pre- and post-exercise both trials) to decrease variation in staining intensity between muscle sections and slides were prepared and analyzed for each participant. For analysis, cryosections were removed from the freezer and fixed immediately in 3.7% formaldehyde for 60 min. Slides were then rinsed with distilled water (3 x 30 s) and treated for 5 min with 0.5% Triton-X100 in phosphate-buffered solution (PBS; 137 mmol·L^{-1} sodium chloride, 3 mmol·L^{-1} potassium chloride, 8 mmol·L^{-1} sodium phosphate dibasic, 3 mmol·L^{-1} potassium phosphate monobasic). The slides were washed (3 x 5 min in PBS) and incubated for 2 h at room temperature with anti-myosin heavy chain I antibody (MHCI; mouse IgM, Developmental Studies Hybridoma Bank: reference #A4.480) and anti-dystrophin antibody (mouse IgG2b, Sigma Aldrich: reference #D8168) in 5% goat serum diluted in PBS (1:1 PBS dilution). This was followed by washes in PBS (3 x 5 min), after which conjugated secondary antibodies [goat anti mouse (GAM) IgM conjugated to AlexaFluor 633 for MHCI; Thermo Fisher: reference #A21046; and GAM IgG2b conjugated to AlexaFluor 594 for dystrophin; Thermo Fisher: reference #A21145] were added and incubated at room temperature (30 min) followed by washes in

PBS. Then, muscle sections were incubated in BODIPY 493/503 solution (Thermo Fisher: reference #D3922) for 20 min at room temperature in a dark room before washes (2 x 3 min in PBS). Stained sections were embedded in Mowiol 4-88 mounting medium (Fluka: reference #81381) and covered with a coverslip. Slides were left to dry overnight at room temperature before analysis by confocal microscope in duplicate (DMIRE2, Leica Microsystems; 40x oil objective; 1.25 NA). An argon laser 488 nm was used to excite BODIPY-493/503 (emission 510-652 nm), while a helium-neon 594 nm and 633 nm laser line were used to excite Alexa Fluor 594 (dystrophin, emission 6680698 nm) and AlexaFluor 633 (MHCI, emission 698-808 nm), respectively. Images were scanned in projection of 4 lines in 1024x1025 pixels format. Quantification of the lipid droplets was performed using Image J software and the intramuscular triglyceride (IMTG) content of each sample was calculated as the percent area of bodipy staining of the total fiber area ([BODIPY stained area [um^2] / area of muscle [um^2]*100).

Muscle glycogen (Acute Study)

Muscle glycogen concentrations were measured using a method described previously (36). Briefly, 10-15 mg of frozen tissue was powdered and transferred into a glass tube pre-cooled on dry ice. Thereafter, the samples were hydrolyzed by adding a 500 µl of 2M HCL and then incubated for 2 h at 95 ºC. After cooling to room temperature, 500µl 2M NaOH was added. Samples were centrifuged and the supernatant was analyzed for glucose concentrations using an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK).

Gene expression (Acute Study)

The mRNA expression of 34 metabolic genes was analyzed using a custom RT2 Profiler PCR Array (Qiagen, USA). First, RNA was extracted from 20-40 mg of powdered muscle tissue using Tri reagent (1 mL, Sigma Aldrich, UK, T9424). After addition of chloroform (200 uL, Acros organics 268320025), tubes were incubated at room temperature for 5 min and centrifuged for 10 min (4 ºC at 12 000 g). The RNA phase was mixed with an equal volume of ice cold 70% ethanol

and RNA was purified on Reliaprep spin columns (Promega, USA, Z6111) as per manufacturer's instructions. The LVis function of the FLUOstar Omega microplate reader was used to measure RNA concentrations to ensure all samples for each participant had the same amount of RNA (184 ng - 400 ng) and samples were reverse transcribed to cDNA using the RT2 First Strand kit (Qiagen, UK, 330401). Quantitative RT-PCR analysis was performed using custom designed 384-well RT2 PCR Profiler Arrays (Qiagen) and RT2 SYBR Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection system (BioRad). 2.8 ng cDNA was added to each well. All primers that were used are commercially available (**Table 2**). The absence of genomic DNA, the efficiency of reverse-transcription and the efficiency of the PCR assay were assessed for each sample and conformed to manufacturer's limits. Relative mRNA expression was determined via the $2^{-\Delta\Delta CT}$ method (37). Housekeeper genes (*β*actin [Refseq# NM_001101]; ribosomal protein lateral stalk subunit P0 [Refseq# NM_001002] and *β*-2-microglobulin [Refseq# NM_004048] were internal controls.

6147

Table 2. List of genes analysed for mRNA expression

Gene	Qiagen catalogue number	Refseq#
CD36	Cat#PPH01356A	NM_000072
SLC27A1	Cat#PPH17902A	NM_198580
SLC27A4	Cat#PPH00471A	NM_005094
FABP3	Cat#PPH02460C	NM_004102
FABP4	Cat#PPH02382F	NM_001442
ACSL1	Cat#PPH19272A	NM_001995
ACSL6	Cat#PPH08013A	NM_001009185
CPT1B	Cat#PPH20905B	NM_001145134
CPT2	Cat#PPH15572A	NM_000098
ACACA	Cat#PPH02316A	NM_000664
ACACB	Cat#PPH02301A	NM_001093
MLYCD	Cat#PPH12795A	NM_012213
HADHA	Cat#PPH10000B	NM_000182
GPAM	Cat#PPH06361A	NM_001244949
DGAT1	Cat#PPH23420F	NM_012079
PNPLA2	Cat#PPH11403B	NM_020376
LIPE	Cat#PPH02383A	NM_005357
PDK4	Cat#PPH07615A	NM_002612
PDK2	Cat#PPH00810A	NM_001199898
GYG1	Cat#PPH13614A	NM_001184720
GYS1	Cat#PPH00988C	NM_001161587
PRKAA1	Cat#PPH00043B	NM_206907
PRKAA2	Cat#PPH15207A	NM_006252
PRKAB2	Cat#PPH09415B	NM_005399
PRKAG1	Cat#PPH07190A	NM_001206709
PPARGC1A	Cat#PPH00461F	NM_013261
PPARA	Cat#PPH01281B	NM_001001928
PPARD	Cat#PPH00455A	NM_001171818
UCP3	Cat#PPH06066A	NM_003356
IL6	Cat#PPH00560C	NM_000600
SLC2A4	Cat#PPH02326A	NM_001042
IRS1	Cat#PPH02328A	NM_005544
IRS2	Cat#PPH02297A	NM_003749
AKT2	Cat#PPH00289F	NM_001243027

Western blotting (Training Study)

For western blots, 40 µg of protein was loaded for each sample and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis on Tris-glycine SDS–polyacrylamide gels (15% for OXPHOS and CPT-1, 10% CD36 and GLUT4 and 8% for AMPK, CHC22, CHC17, AkT and AS160). Gels were electro-blotted (semi-dry transfer) onto a nitrocellulose membrane and were then washed in Tris-buffered saline (0.09% NaCl, 100 mM Tris–HCl pH 7.4) with 0.1% Tween 20 (TBS-T) and incubated for 30 min in a blocking solution (5% non-fat milk in TBS-T). Membranes were incubated overnight at 4 °C with primary antibodies against OXPHOS (Abcam: reference #ab110411), CPT-1 (Abcam: reference #ab134988), CD36 (Abcam: reference #ab133625), GLUT4 [self-raised rabbit polyclonal antibody against the C-terminus of GLUT4 (38)], CHC22 [SHL-KS, affinity purified self-raised rabbit polyclonal against the CHC22 C-terminus cross-absorbed against the CHC17 C-terminus (39)], CHC17 [TD.1 self-raised mouse monoclonal against CHC17 terminal domain (40)] AMPKα (Cell Signalling Technologies: reference #2532), Akt (Cell Signaling Technologies: reference #3063), AS160 (Millipore: reference #07-741). It should be noted that the AMPKα antibody recognises both α1 and AMPKα2 isoforms of the catalytic subunit and does not detect the regulatory AMPKβ or AMPKγ subunits (41). Following incubation with the primary antibodies, the membranes were washed in TBS-T and incubated for 60 min in a 1:4000 dilution of anti-species IgG horseradish peroxidase-conjugated secondary antibodies in the aforementioned blocking solution. After further washes, membranes were incubated in an enhanced chemiluminescence reagent and visualized (EpiChemi II Darkroom, UVP, Upland, USA). The band densities were quantified using Image Studio Lite software (Version 5.2; LI-COR, Nebraska, USA) and were normalized to either GAPDH (Proteintech: reference #60004-1-Ig) or Actin (Sigma Aldrich: reference #A2066), before the pre- to post-intervention change was calculated. Pre- and post-intervention samples from any given participant were included on the same gel. Citrate synthase activity was measured using a commercially available assay (Abcam: reference #ab119692).

Phospholipid composition (Training Study)

Samples were freeze-dried, powdered under liquid nitrogen, and transferred into glass tubes containing 2 ml of methanol and butylated hydroxytoluene (0.01%) and heptadecanoic acid (as an internal standard), followed by the addition of 4 mL of chloroform and 1.5 mL of water, before lipids were extracted. The lipid containing fraction was transferred into thin-layer chromatography (TLC; Kieselgel 60, 0.22 mm, Merck, Darmstadt, Germany) silica plates and lipids were separated by TLC with a heptane: isopropyl ether: acetic acid (60:40:3, vol/vol/vol) resolving solution. Lipid bands were made visible by spraying the plates with a 0.2% solution of 3'7'-dichlorofluorescin in methanol and recognized under ultraviolet light using standards on the plates. Then the gel bands containing phospholipids were scraped off the plates, transferred into screw cap tubes and transmethylated with BF3/methanol. The fatty acid methyl esters (FAMEs) were then dissolved in hexane and analyzed by GLC. A Hewlett-Packard 5890 Series II gas chromatograph with Varian CP-SIL capillary column (100 m, internal diameter of 0.25 mm) and flame-ionization detector were used. In accordance with the retention times of standards, the individual long-chain fatty acids quantification was performed. The content of phospholipids was estimated as the sum of the total fatty acid species and expressed in nanomoles per milligram of dry mass (42,43).

Energy expenditure and intake (Training Study)

Average daily energy expenditure was calculated as the sum of the resting metabolic rate (RMR), diet-induced thermogenesis (10% of self-reported daily energy intake) and physical activity energy expenditure (PAEE). To assess RMR, participants rested in a semi-supine position for 15 min before 4 x 5-min expired air samples were collected (44). The participants were provided with the mouthpiece 1 min prior to sample collections (as a stabilization period) which were collected into a 200-L Douglas bag (Hans Rudolph, Kansas City, USA) via falconia tubing (Baxter, Woodhouse and Taylor Ltd, Macclesfield, UK). Concurrent measures of inspired air were also made to correct for changes in the ambient O_2 and CO_2 concentrations. Expired O_2 and CO_2 concentrations were

measured in a volume of each sample using paramagnetic and infrared transducers (Mini HF 5200, Servomex Group Ltd., Crowborough, UK). The sensor was calibrated with low (0% O_2 and 0% CO_2) and high (16.04% O_2, 5.06% CO_2) calibration gases (BOC Industrial Gases, Munich, Germany). Substrate utilization rates were then calculated via stochiometric equations (45,46). Energy expenditure was calculated assuming that fatty acids, glucose and glycogen provide 40.81 $kJ \cdot g^{-1}$, 15.64 $kJ \cdot g^{-1}$ and 17.36 $kJ \cdot g^{-1}$ of energy, respectively. To measure free-living PAEE, participants wore an Actiheart™ monitor over 7 days (Cambridge Neurotechnology, Papworth, UK). This monitor integrates accelerometry and heart rate signals and has been validated as a measure of energy expenditure (47-49). Energy expenditure and heart rate values from rest and exercise were entered in the Actiheart™ software for an individually calibrated model. Participants were also asked to keep a written record of their food and fluid intake for 4 days over a typical 7-day period (including a weekend day) pre- and during the last week of the intervention. Weighing scales were provided to increase the accuracy of records. Records were analyzed using Nutritics software (Nutritics Ltd., Dublin, Ireland). The macronutrient composition of each food was taken from the manufacturer's labels, but if this was not possible (e.g. fresh products) foods were analyzed via the software database or comparable brands were used to provide the relevant information, and this was kept constant across records.

Statistics

In the **Acute Study**, the sample size was based upon data demonstrating an attenuation of intramuscular lipid utilization during exercise with carbohydrate intake before and during exercise with an effect size of d = 1.5. (22) We aimed to recruit 12 participants assuming at least 8 participants would complete the study with biopsies to provide >90% power with α set at 0.05. In the **Training Study**, a sample size estimation was completed using data from a training study in healthy, lean men (26). In that study, a change in the plasma glucose AUC for an oral glucose tolerance test (OGTT) of -65±53 $mmol \cdot min \cdot L^{-1}$ was shown in an EX-BR group *versus* +21±47 $mmol \cdot min \cdot L^{-1}$ for a CON group. With α set at 0.05, 9

participants were required for a >90% chance of detecting this effect. We therefore recruited 30 participants to account for the possibility of an unequal allocation of participants across three groups when using a stratified randomization schedule. Participants were allocated to the CON (*n*=9), BR-EX (*n*=12) or EX-BR (*n*=9) groups using this schedule, which was generated by JPW and included a factor for Physical Activity Level (PAL) and the time-averaged glucose AUC for the baseline OGTT which was assessed using a Freestyle Freedom Lite Glucose Meter. This was to ensure an even distribution of less (PAL <1.65) and more active (PAL >1.65) participants and participants with glucose AUC values above or below 8 $mmol \cdot L^{-1}$. Data are presented as means ± 95% confidence intervals (CI), except for participant characteristics (which are mean ± SD). A Shapiro-Wilk test was performed to test for normal distribution and if this was not obtained, non-parametric tests (e.g. Wilcoxon matched-pairs signed rank tests) were employed. In the **Acute Study**, differences between groups were assessed with paired *t*-tests or a two-way repeated measures ANOVA (for variables dependent on time). In the **Training Study**, one-way ANOVAs were used to assess differences between groups at baseline and two-way mixed-design ANOVAs were used to assess differences between groups in response to the intervention (group x time). If interaction effects were identified, independent *t*-tests were used to locate variance, with Holm-Bonferroni step-wise adjustments made. Correlations between variables were explored using Pearson *r* or Spearman R for normal or non-normal distributions, respectively. A significance level of $p<0.05$ was always used. The area under the concentration-time curve (AUC) was calculated via the trapezoid rule and divided by the duration of an observation period of interest for a time-averaged summary value. Plasma glucose and insulin concentrations were used to assess oral glucose insulin sensitivity (the OGIS index) as per instructions provided at; http://webmet.pd.cnr.it/ogis/ (50). Statistical analyses were completed on IBM SPSS statistics V 22 for windows (except for the Holm-Bonferroni adjustments which were completed on Microsoft Excel) and Graph Pad Prism 7 was used to prepare the figures. As we were unable to collect data from all participants for all measured outcomes the *n* are always displayed in all figure and table captions.

RESULTS

Exercise before nutrient ingestion increases whole-body and skeletal muscle lipid utilization but does not differentially modulate muscle gene expression

In the **Acute Study**, exercising before *versus* after nutrient provision increased the acute plasma glucose and serum insulin responses to food consumption (**Figure 2A** and **2B**). The plasma glucose AUC was 6.70 [6.00 to 7.39] mmol·L^{-1}·330 min^{-1} with exercise before nutrient provision *versus* 5.91 [5.33 to 6.50] mmol·L^{-1}·330 min^{-1} with exercise after nutrient provision (p<0.01). The serum insulin AUC was 86.9 [48.5 to 125.2] pmol·L^{-1}·330 min^{-1} with exercise before nutrient provision *versus* 55.3 [31.2 to 79.3] pmol·L^{-1}·330 min^{-1} with exercise after nutrient provision (p<0.01). Exercise performed before *versus* after nutrient provision resulted in higher glycerol and non-esterified fatty acid (NEFA) concentrations during the exercise (**Figure 2C** and **2D**).

Nutrient provision before exercise potently altered whole-body metabolism, resulting in an increase in whole-body carbohydrate utilization (**Figure 2E**) and a decrease in whole-body lipid utilization (**Figure 2F**). In skeletal muscle, glycogen utilization during exercise (time effect, p<0.01) was independent of nutrient-exercise timing (time x trial interaction effect p=0.12; **Figure 2G**). However, the type I muscle fibre intramuscular triglyceride (IMTG) content was only reduced with exercise performed before nutrient provision (time x trial interaction: p=0.02; **Figure 2H and 2J**). A similar pattern was observed for the type II muscle fibre IMTG content (time x trial interaction: p=0.04; **Figure 2I and 2J**), although the reduction with exercise before nutrient provision did not achieve statistical significance after post-hoc corrections. Nonetheless, clear differences (both p<0.05) in the net changes in the IMTG content were observed in both fibre types with exercise before *versus* after nutrient provision (for type I: -3.44 [-1.61 to -5.26]% *versus* 1.44 [-1.46 to 4.34]% area covered by lipid staining and type II: -1.89 {-0.16 to -3.61]% *versus* 1.83 [0.50 to 3.17]% area covered by lipid staining for exercise before *versus* after nutrient provision, respectively).

Of the 34 selected genes that are implicated in metabolic adaptations to exercise, only 8 genes were altered by exercise, whereby *IRS-1* and *FATP1* were decreased post-exercise compared to baseline ($p<0.05$) and *IRS-2, PDK4, PGC1α, FATP4* and *ACSL1* were increased post-exercise compared to baseline (all $p<0.05$). However, only *PPARδ* was differentially expressed by nutrient-exercise timing and was higher with breakfast before *versus* after exercise ($p<0.05$; **Figure 3**).

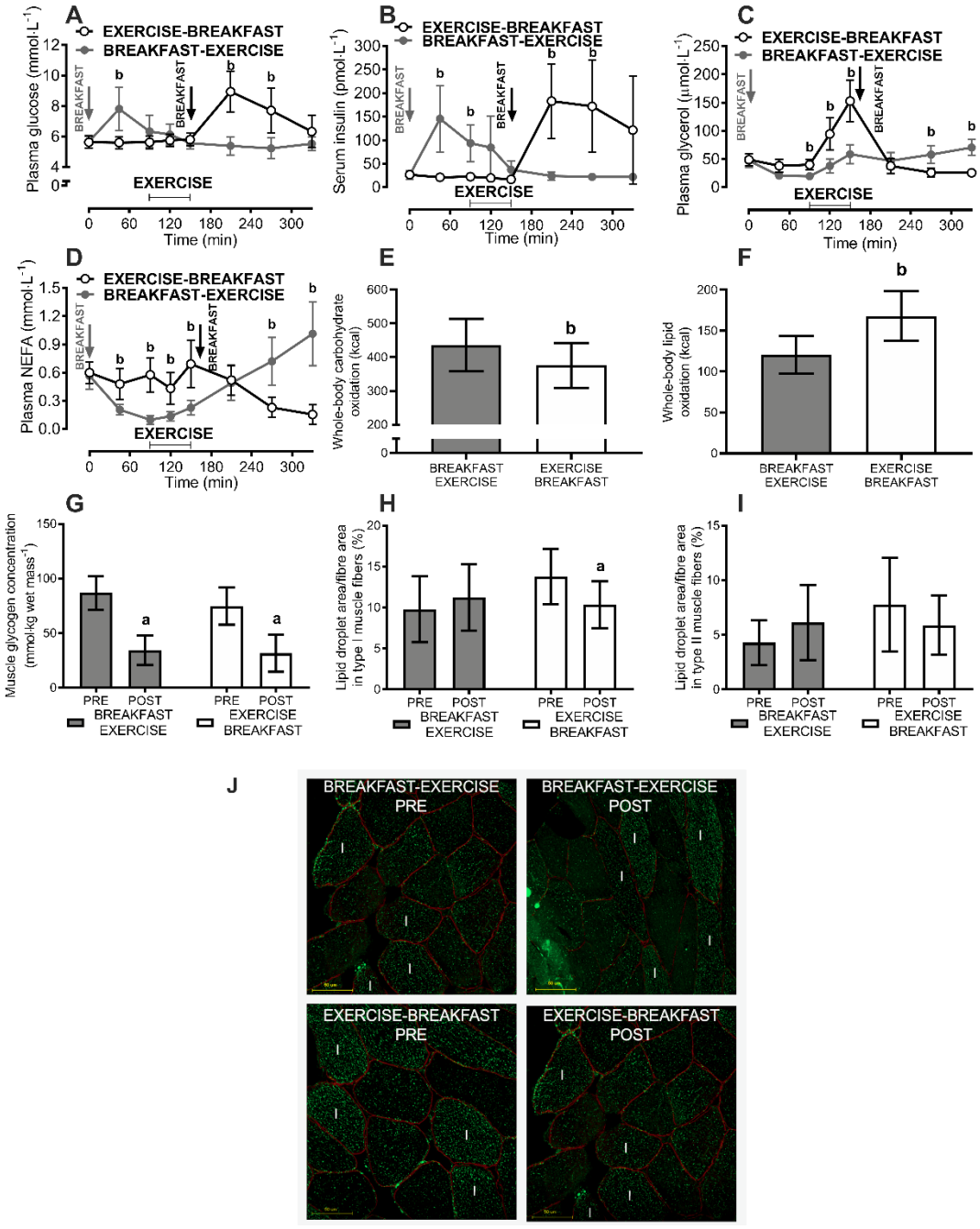
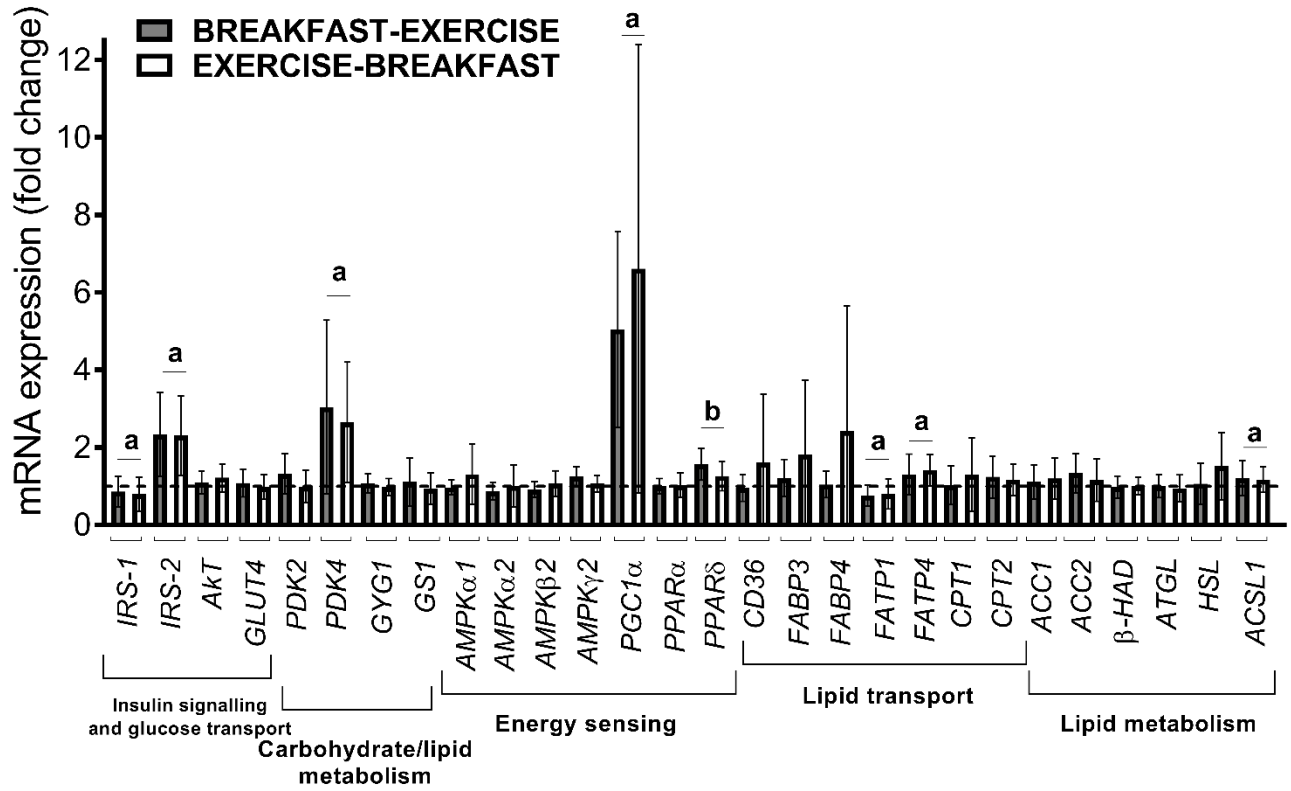


Figure 2. Plasma glucose (**A**), serum insulin (**B**), plasma glycerol (**C**) and plasma NEFA (**D**) concentrations and whole-body carbohydrate (**E**) and fat (**F**) utilization. Muscle was sampled pre- and post-exercise (*vastus lateralis*) to assess mixed-muscle glycogen (**G**) and fiber-type specific intramuscular lipid (IMTG) utilization (**H & I**). Panel **J** is images where IMTG (stained green) in combination with dystrophin (to identify the cell border, stained red) is shown from muscle samples of a representative participant for breakfast-exercise and exercise-breakfast trials. White I shows type 1 fibers and all other fibers are assumed to be type II. Yellow bars are scale (50 μm). All data are presented as means ± 95% CI. For panels **A-F** *n*=12, for panels **G**, **H and I** *n*=9. [a] difference with pre- *versus* post-exercise; [b] breakfast-exercise *versus* exercise-breakfast ($p < 0.05$).



6326
6327 **Figure 3.** Skeletal muscle mRNA expression responses to a bout of exercise
6328 before *versus* after nutrient provision (in the form of breakfast) in overweight men.
6329 *n*=8. Muscle was sample pre- and at 3 h post-exercise (*vastus lateralis*) to assess
6330 the intramuscular gene expression responses to exercise. [a]effect of exercise; [b]
6331 difference between breakfast-exercise *versus* exercise-breakfast ($p < 0.05$).

Exercise training before nutrient provision leads to sustained increases in lipid utilization

In the **Training Study** the compliance to the training was 100%, as all sessions were completed as prescribed. The average exercise intensity was 62 ± 5% $\dot{V}O_2$ peak in BR-EX and 62 ± 4% $\dot{V}O_2$ peak in EX-BR (p=0.98) and the heart rate (HR) response and average rating of perceived exertion (RPE) to the exercise training were 140 ± 13 *versus* 134 ± 8 beats·min^{-1} in BR-EX *versus* EX-BR (p=0.18) and 13 ± 1 au *versus* 13 ± 1 au (6-20 rating scale) in BR-EX *versus* EX-BR; (p=0.54), respectively. In the **Training Study** rates of whole-body lipid utilization were around 2-fold higher with exercise before *versus* after nutrient provision and this difference between the conditions was sustained throughout the whole 6-week intervention (**Figure 4A**). As a consequence, regular exercise before (*versus* after) nutrient provision increased cumulative whole-body lipid utilization (during exercise) over a 6-week intervention, from 799 kcal [530 to 1069] in BR-EX to 1666 kcal [1260 to 2072] in EX-BR (p<0.01). This was accompanied by a decrease in whole-body carbohydrate utilization during exercise (**Figure 5A**), as reflected by a decrease in the respiratory exchange ratio (group effect, p<0.01; **Figure 5B**). However, cumulative energy expenditure throughout the exercise intervention did not differ with exercise performed before *versus* after nutrient provision (**Figure 5C**; 7207 [6739 to 7676] kcal in BR-EX *versus* 6951 [6267 to 7635] kcal in EX-BR; p=0.48).

Exercise training before nutrient provision leads to increased oral glucose insulin sensitivity

The oral glucose tolerance test-derived estimate of peripheral insulin sensitivity (the OGIS index; p=0.26), postprandial glycaemia (p=0.80) and postprandial insulinemia (p=0.30) were similar between groups pre-intervention (**Table 3**). The intervention-induced changes in postprandial glycaemia (time x group interaction, p=0.54; **Figure 4B**) and fasting blood lipid profiles were unaffected by nutrient-exercise timing (pre- and post-intervention data are shown in **Tables 3 and 4**). However, exercise training before, but not after nutrient intake reduced postprandial insulinemia (time x group interaction p=0.03; **Figure 4C)**. Exercise training before *versus* after nutrient provision also increased the OGIS index (time

x group interaction p=0.03; **Figure 4D** with pre- and post-intervention data in **Table 3**). The plasma C-peptide-to-insulin ratio was not differentially altered by nutrient-exercise timing (time x group interaction, p=0.12; **Figure 4E**). The change in the OGIS index in response to exercise training was positively and moderately correlated with cumulative lipid utilization during exercise during the intervention (**Figure 4F**) but not with cumulative energy expenditure (**Figure 5C**).

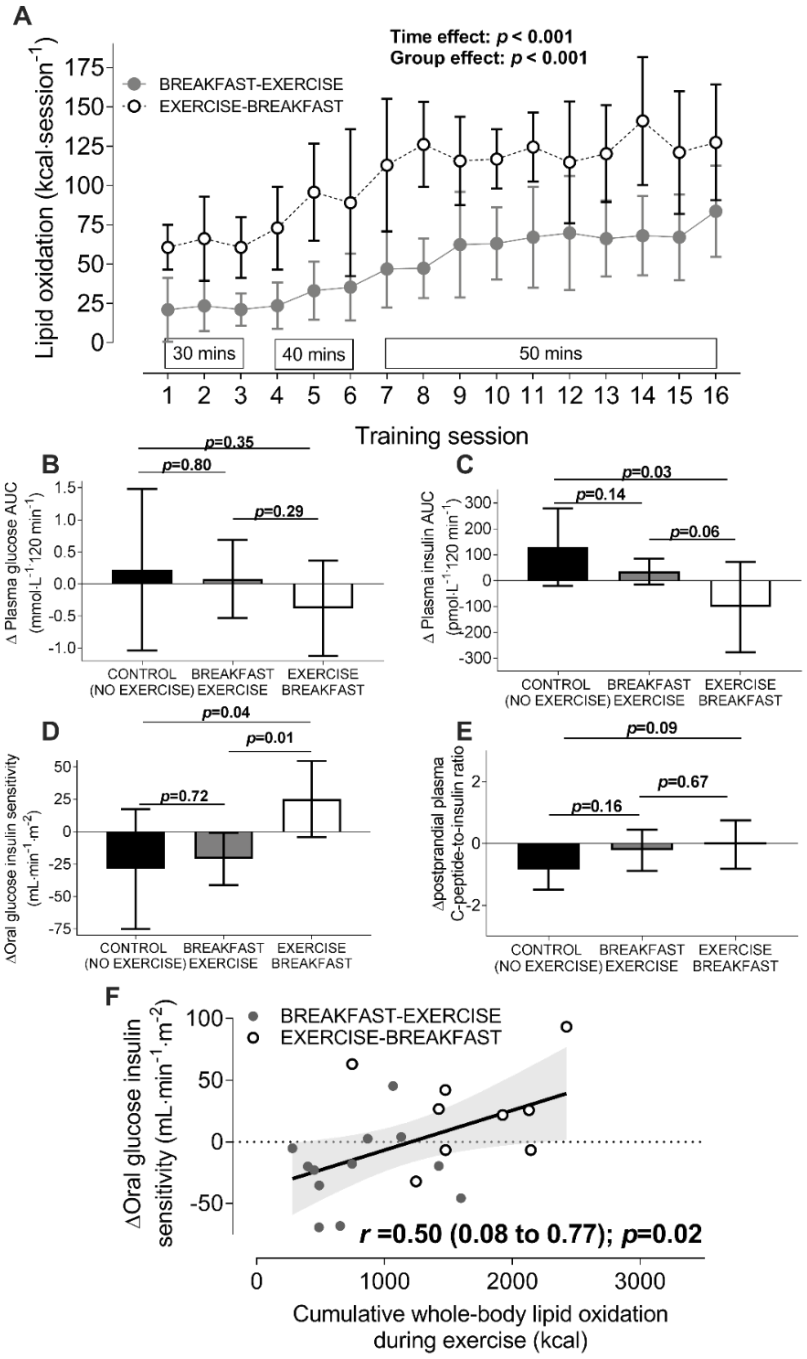


Figure 4. Whole-body lipid utilization during every a 6-week exercise intervention (**A**) and the change in the plasma glucose AUC (**B**), plasma insulin AUC (**C**), the oral glucose insulin sensitivity index (OGIS; **D**) and the postprandial plasma C-peptide: insulin ratio (**E**) in control, breakfast-exercise and exercise-breakfast groups. Panel **F** is a Pearson correlation between changes in the OGIS index and cumulative lipid utilization throughout the training intervention. All data are means ± 95% CI. For control n = 9, for breakfast-exercise n = 12 and for exercise-breakfast n = 9 men classified as overweight or obese. The shaded area is the 95% confidence bands for the regression line. [a] difference between control *versus* exercise-breakfast; [b] breakfast-exercise *versus* exercise-breakfast ($p < 0.05$).

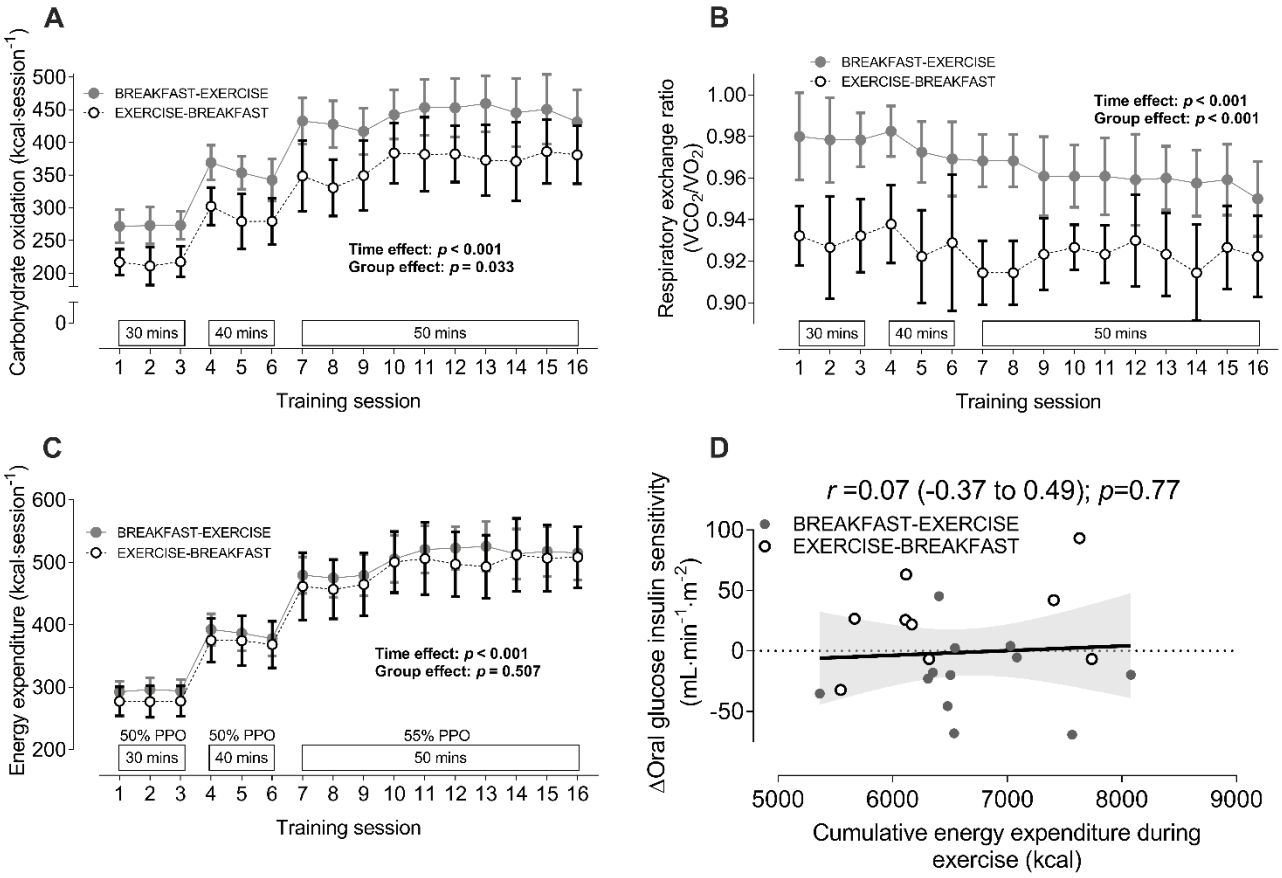


Figure 5. Energy expenditure (**A**), the respiratory exchange ratio (**B**) and whole-body carbohydrate utilisation rates (**C**) during every exercise session in a 6-week training intervention and a Pearson correlation between cumulative energy expenditure throughout the exercise training intervention with the changes in oral glucose insulin sensitivity (the OGIS index) with exercise before *versus* after nutrient intake (**D**). All data are presented as means ± 95% CI. For control n = 9, for breakfast-exercise n = 12 and for exercise-breakfast n = 9 men classified as overweight or obese. The shaded grey area represents the 95% confidence bands for the regression line.

Table 3. Postprandial plasma metabolite concentrations for the control (CON; *n*=9), breakfast-exercise (BR-EX; *n*=12) and exercise-breakfast (EX-BR; *n*=9) groups.

	Pre-intervention	**Post-intervention**	
CON glucose AUC (mmol·L^{-1})	8.20 (1.36)	8.43 (0.89)	*0.69*
BR-EX glucose AUC (mmol·L^{-1})	8.26 (0.90)	8.34 (1.35)	*0.78*
EX-BR glucose AUC (mmol·L^{-1})	8.52 (1.05)	8.14 (1.03)	*0.27*
CON insulin AUC (pmol·L^{-1})	***385 (221)***	***514 (382)***	***0.08***
BR-EX insulin AUC (pmol·L^{-1})	***268 (109)***	***303 (135)***	***0.16***
EX-BR insulin AUC (pmol·L^{-1})	***458 (441)***	***355 (254)***	***0.21***
CON C-peptide AUC (ng·mL^{-1})	6.59 (2.57)	7.32 (3.11)	*0.18*
BR-EX C-peptide AUC (ng·mL^{-1})	5.25 (1.72)	5.54 (1.87)	*0.34*
EX-BR C-peptide AUC (ng·mL^{-1})	6.81 (4.40)	6.03 (3.45)	*0.28*
CON NEFA AUC (mmol·L^{-1})	***0.17 (0.07)***	***0.19 (0.07)***	***0.52***
BR-EX NEFA AUC (mmol·L^{-1})	***0.20 (0.08)***	***0.16 (0.06)***	***0.08***
EX-BR NEFA AUC (mmol·L^{-1})	***0.14 (0.03)***	***0.13 (0.04)***	***0.48***
CON OGIS (mL·min^{-1}·m^{-2})	401 (39)	372 (63)	*0.19*
BR-EX OGIS (mL·min^{-1}·m^{-2})	403 (31)	380 (34)	*0.06*
EX-BR OGIS (mL·min^{-1}·m^{-2})	374 (56)	399 (42)	*0.08*

Data are means and (SD). Abbreviations: NEFA = non-esterified fatty acid, OGIS = oral glucose insulin sensitivity, AUC = time-averaged area under the curve for the OGTT (120 min).

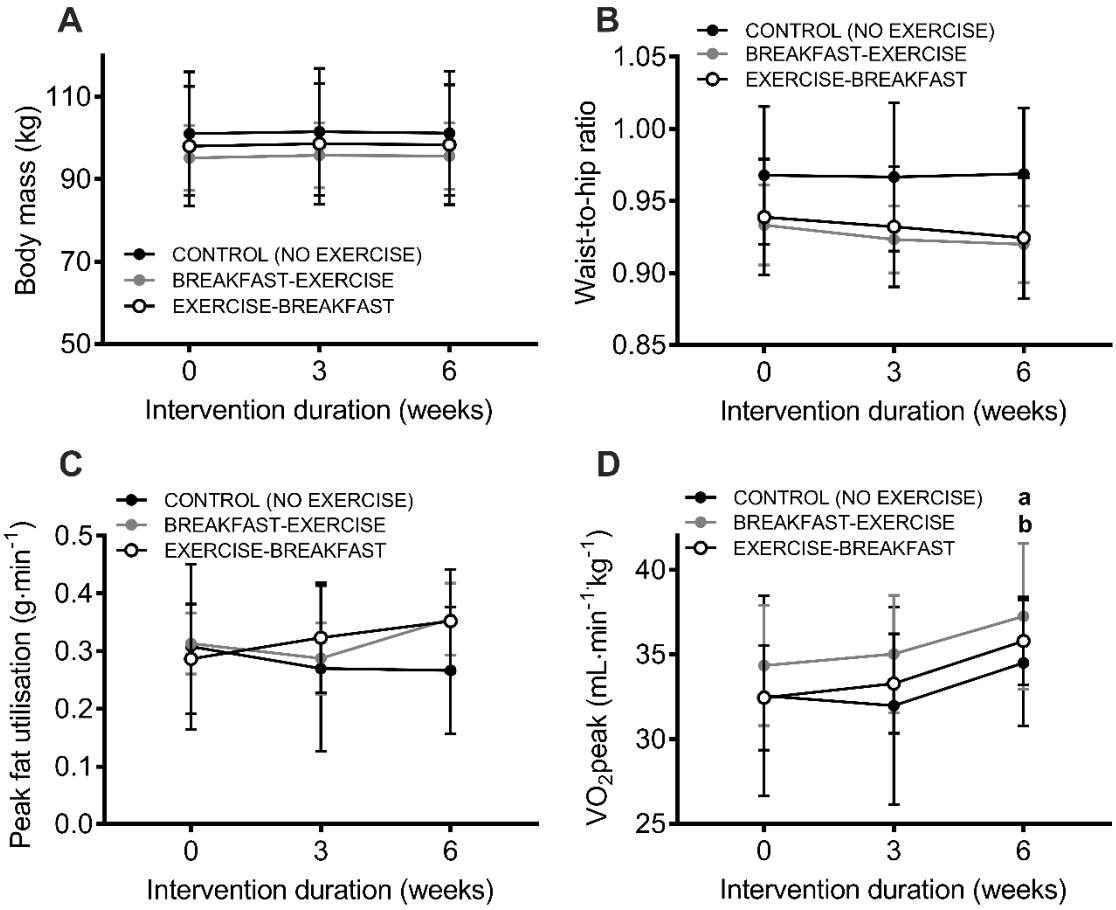
Table 4. Fasting plasma metabolite concentrations for the control (CON; *n*=9), breakfast-exercise (BR-EX; *n*=12) and exercise-breakfast (EX-BR; *n*=9) groups.

	Pre-intervention	Post-intervention	Δ from pre-intervention	*time x group interaction*
CON glucose (mmol·L^{-1})	5.39 (0.49)	5.57 (0.66)	0.17 (-0.32, 0.66)	*F*=1.413 *p*= 0.26
BR-EX glucose (mmol·L^{-1})	5.48 (0.33)	5.60 (0.47)	0.12 (-0.30, 0.54)	
EX-BR glucose (mmol·L^{-1})	5.72 (0.71)	5.46 (0.72)	-0.27 (-0.67, 0.13)	
CON insulin (pmol·L^{-1})	95 (121)	81 (63)	-14 (-7, 8)	F=0.327 p= 0.72
BR-EX insulin (pmol·L^{-1})	43 (23)	43 (24)	0 (-14, 15)	
EX-BR insulin (pmol·L^{-1})	49 (43)	47 (35)	-2 (-20, 14)	
CON HOMA-IR (au)	0.35 (0.04)	0.34 (0.03)	-0.01 (-0.02, 0.00)	F=0.458 p=0.40
BR-EX HOMA-IR (au)	0.37 (0.03)	0.37 (0.04)	0.00 (-0.01, 0.01)	
EX-BR HOMA-IR (au)	0.37 (0.04)	0.37 (0.04)	0.01 (-0.02, 0.04)	
CON NEFA (mmol·L^{-1})	0.36 (0.16)	0.39 (0.11)	0.03 (-0.10, 0.15)	F=1.021 p=0.37
BR-EX NEFA (mmol·L^{-1})	0.44 (0.15)	0.39 (0.10)	-0.05 (-0.11, 0.15)	
EX-BR NEFA (mmol·L^{-1})	0.34 (0.09)	0.32 (0.10)	-0.02 (-0.09, 0.05)	
CON TAG (mmol·L^{-1})	1.59 (0.77)	2.05 (0.70)	0.46 (-0.06, 0.98)	F= 5.967 p<0.01
BR-EX TAG (mmol·L^{-1})	1.34 (0.84)	1.11 (0.42)	-0.24 (-0.55, 0.76)a	
EX-BR TAG (mmol·L^{-1})	1.10 (0.31)	0.88 (0.36)	-0.22 (-0.41, -0.03)b	
CON cholesterol (mmol·L^{-1})	4.41 (1.23)	4.59 (1.36)	0.19 (-0.74, 1.12)	F=1.707 p=0.20
BR-EX cholesterol (mmol·L^{-1})	3.77 (1.34)	3.72 (1.21)	-0.05 (-0.41, 0.30)	
EX-BR cholesterol (mmol·L^{-1})	3.74 (0.82)	3.24 (0.92)	-0.51 (-0.95, -0.07)	
CON HDL cholesterol (mmol·L^{-1})	0.84 (0.17)	0.86 (0.20)	0.02 (-0.14, 0.18)	F=1.634 p=0.21
BR-EX HDL cholesterol (mmol·L^{-1})	0.82 (0.31)	0.84 (0.33)	0.02 (-0.05, 0.10)	
EX-BR HDL cholesterol (mmol·L^{-1})	0.90 (0.23)	0.81 (0.26)	-0.09 (-0.19, 0.01)	
CON LDL cholesterol (mmol·L^{-1})	3.36 (1.33)	3.56 (1.36)	0.20 (-0.50, 0.89)	F=2.110 p=0.14
BR-EX LDL cholesterol (mmol·L^{-1})	2.71 (0.97)	2.70 (0.99)	-0.01 (-0.31, 0.28)	
EX-BR LDLcholesterol (mmol·L^{-1})	2.72 (0.57)	2.31 (0.58)	-0.41 (-0.80, -0.02)	

Data are means and (SD) except for change scores which are means and (95% CI). Abbreviations: HOMA-IR = the homeostatic model of insulin resistance, NEFA = non-esterified fatty acid, TAG = triglyceride, HDL = high density lipoprotein, LDL = low density lipoprotein a = difference in change from pre- to post-intervention for CON versus BR-EX and b for CON versus EX-BR with p<0.05.

Nutrient-exercise timing does not differentially alter body composition or oxidative capacity

Exercise before *versus* after nutrient provision resulted in comparable changes in body mass (time x group interaction, p=0.97; **Figure 6A**), a marker of central adiposity (the waist to hip ratio; time x group interaction, p=0.17, **Figure 6B**), and the peak capacity for whole-body lipid utilization (time x group interaction, p=0.14; **Figure 6C**). Exercise training increased $\dot{V}O_2$ peak by ~ 3 mL·kg·min^{-1} relative to a no-exercise control (CON) group (time x group interaction, p=0.01) but the magnitude of this increase in cardiorespiratory fitness was unaffected by nutrient exercise timing (p=0.54 with breakfast-exercise *versus* exercise-breakfast). Self-reported daily energy intake was unaffected by exercise or nutrient-exercise timing (time x group interaction, p=0.38; **Table 5**), and although daily energy expenditure was increased in the exercise groups *versus* control group (time x group interaction, p=0.01; **Table 5**), this increase was unaffected by nutrient-exercise timing (p=0.38).



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6410 **Figure 6.** Body mass (**A**), the waist-to-hip ratio (**B**), whole-body oxidative capacity
6411 (VO_2peak; **C**) and peak fat utilization rates during an incremental exercise test
6412 (**D**) at baseline, week 3 and week 6 of an intervention in control (no-exercise),
6413 breakfast-exercise and exercise-breakfast groups. All data are presented as
6414 means ± 95% CI. For control *n* = 9, for breakfast-exercise *n* = 12 and for exercise-
6415 breakfast *n* = 9 men classified as overweight or obese [a] difference between
6416 control *versus* breakfast-exercise; [b] control *versus* exercise-breakfast ($p < 0.05$).

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Table 5. Components of daily energy intake and daily energy expenditure pre- and post-intervention for the control (CON; n=9 [except for PAEE where *n*=8]), breakfast before exercise (BR-EX; n=12 [except for PAEE where *n*=10]) and exercise before breakfast (EX-BR; n=9) groups.

	Pre-intervention	Post-intervention	Δ from pre-intervention	*time x group interaction*
CON RMR (kcal·d^{-1})	1997 (225)	2021 (232)	24 (-56, 104)	F=1.791 p=0.19
BR-EX RMR (kcal·d^{-1})	1964 (167)	2074 (208)	110 (45, 174)	
EX-BR RMR (kcal·d^{-1})	1899 (228)	1967 (194)	68 (-13, 148)	
CON TEF (kcal·d^{-1})	283 (61)	292 (85)	9 (-29, 48)	F=0.982 p= 0.38
BR-EX TEF (kcal·d^{-1})	292 (50)	288 (42)	-4 (-19, 10)	
EX-BR TEF (kcal·d^{-1})	271 (55)	258 (44)	-13 (-29, 4)	
CON PAEE (kcal·d^{-1})	1144 (298)	1077 (327)	-67 (-215, 81)	F=7.044 p<0.01
BR-EX PAEE (kcal·d^{-1})	986 (264)	1190 (343)	204 (56, 352) a	
EX-BR PAEE (kcal·d^{-1})	1006 (147)	1357 (324)	351 (126, 576) b	
CON CHO intake (kcal·d^{-1})	1180 (357)	1424 (600)	244 (-29, 517)	F=0.977 p=0.39
BR-EX CHO intake (kcal·d^{-1})	1171 (317)	1272 (220)	101 (-51, 252)	
EX-BR CHO intake (kcal·d^{-1})	1133 (307)	1214 (234)	81 (-91, 252)	
CON FAT intake (kcal·d^{-1})	1148 (326)	1045 (311)	-104 (-257, 49)	F=0.347 p=0.71
BR-EX FAT intake (kcal·d^{-1})	1132 (307)	1091 (238)	-41 (-156, 75)	
EX-BR FAT intake (kcal·d^{-1})	987 (320)	882 (172)	-105 (-289, 79)	
CON PRO intake (kcal·d^{-1})	429 (97)	376 (89)	-52 (-89, 15)	F=1.517 p=0.24
BR-EX PRO intake (kcal·d^{-1})	511 (122)	446 (59)	-65 (-118, -11)	
EX-BR PRO intake (kcal·d^{-1})	406 (97)	399 (117)	-7 (-75, 62)	
CON ALC intake (kcal·d^{-1})	72 (95)	75 (154)	3 (-145, 152)	F=1.115 p=0.34
BR-EX ALC intake (kcal·d^{-1})	111 (118)	69 (72)	-42 (-101, 17)	
EX-BR ALC intake (kcal·d^{-1})	185 (168)	90 (131)	-94 (-191, 2)	

Data are means and (SD) except for change scores which are means and (95% CI). Abbreviations: CHO = carbohydrate, PRO = protein, ALC = alcohol, RMR = resting metabolic rate, TEF = thermic effect of feeding, PAEE = physical activity energy expenditure. [a] denotes a difference in the change from pre-to post-intervention for CON *versus* BR-EX and [b] for CON *versus* EX-BR with $p<0.05$.

Exercise training before nutrient provision increases phospholipid remodeling

There was a significant effect on global skeletal muscle remodeling with exercise before *versus* after nutrient ingestion as quantified by the sum of changes in the fatty acid content of all phospholipid species (p=0.01; **Figure 7A**). Nutrient provision prior to exercise prevented this exercise-induced increase in skeletal muscle phospholipid remodeling as the sum of changes was not different to a non-exercise control group (p=0.41). No clear time x group interaction effects were determined for any of the measured fatty acid species, except for the proportion of 18:0, which increased with exercise before nutrient provision compared to the control group (**Table 6**). The change in the overall saturated fatty acid content of skeletal muscle phospholipids was moderately and positively correlated with changes in postprandial insulinemia and the relationship was robust to the exclusion of any single data point (**Figure 7B**).

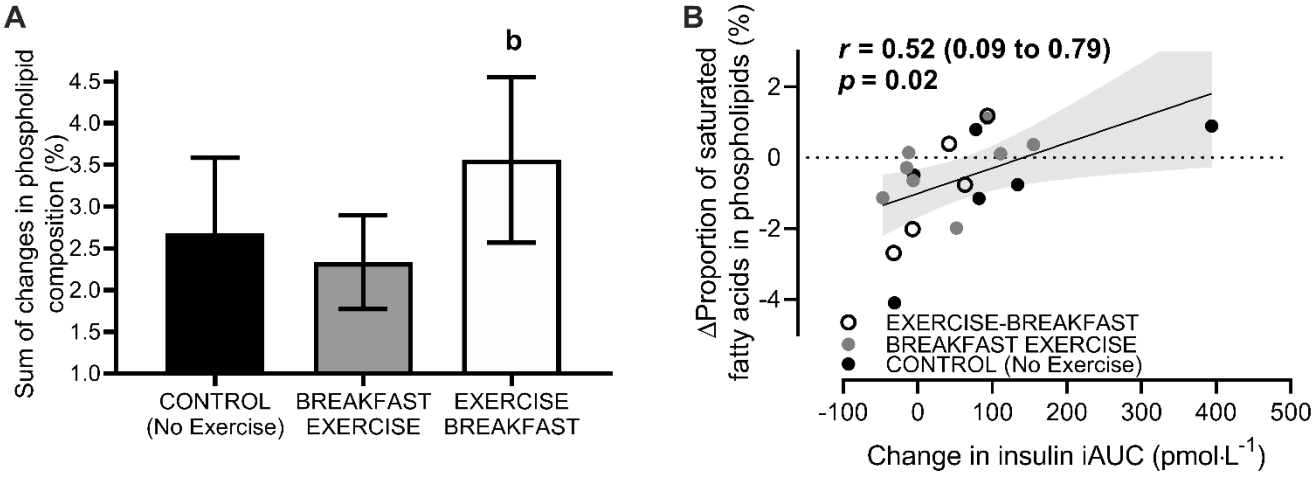

Figure 7. Pre-to-post intervention changes in the sum of all changes in the fatty acid content of phospholipid species (**A**) and a Pearson correlation between postprandial insulinemia with the change in the proportion of saturated fatty acids in skeletal muscle phospholipids (**B**). All data are presented as means ± 95% CI. For control n = 6, for breakfast-exercise n = 9 and for exercise-breakfast n = 5 men classified as overweight or obese. The shaded area represents the 95% confidence bands for the regression line. [b] difference between breakfast-exercise *versus* exercise-breakfast (p < 0.05).

Table 6. Skeletal muscle phospholipid composition in a control (CON; *n*=6), breakfast-exercise (BR-EX; *n*=8) and exercise-breakfast (EX-BR; *n*=5) groups.

	Pre-intervention	Post-intervention	*time x group interaction*
CON 14:0 (% of total)	1.3 (0.5)	1.0 (0.3)	F=0.493 p=0.62
BR-EX 14:0 (% of total)	0.8 (0.1)	0.7 (0.1)	
EX-BR 14:0 (% of total)	0.8 (0.4)	0.7 (0.1)	
CON 16:0 (% of total)	24.8 (2.3)	24.3 (1.0)	F=1.537 p=0.25
BR-EX 16:0 (% of total)	22.4 (0.6)	21.7 (1.2)	
EX-BR 16:0 (% of total)	19.5 (1.5)	17.9 (1.4)	
CON 18:0 (% of total)	14.2 (0.3)	14.2 (0.5)	F=7.205 p<0.01
BR-EX 18:0 (% of total)	14.6 (0.4)	15.2 (0.6)	
EX-BR 18:0 (% of total)	11.9 (0.8)	13.0 (0.8) [a]	
CON 20:0 (% of total)	0.13 (0.05)	0.09 (0.02)	F=1.141 p=0.34
BR-EX 20:0 (% of total)	0.08 (0.02)	0.08 (0.04)	
EX-BR 20:0 (% of total)	0.10 (0.04)	0.10 (0.07)	
CON 22:0 (% of total)	0.21 (0.07)	0.20 (0.02)	F=0.666 p=0.53
BR-EX 22:0 (% of total)	0.20 (0.04)	0.19 (0.04)	
EX-BR 22:0 (% of total)	0.20 (0.05)	0.23 (0.10)	
CON 24:0 (% of total)	0.15 (0.07)	0.14 (0.04)	F=0.108 p=0.90
BR-EX 24:0 (% of total)	0.12 (0.08)	0.11 (0.06)	
EX-BR 24:0 (% of total)	0.16 (0.08)	0.13 (0.06)	
CON 16:1 (% of total)	0.82 (0.34)	0.70 (0.19)	F=1.254 p=0.31
BR-EX 16:1 (% of total)	0.66 (0.12)	0.75 (0.26)	
EX-BR 16:1 (% of total)	0.66 (0.15)	0.62 (0.17)	
CON 18:1n9c (% of total)	7.9 (3.0)	7.1 (1.5)	F=2.970 p=0.08
BR-EX 18:1n9c (% of total)	6.3 (0.6)	6.8 (0.6)	
EX-BR 18:1n9c (% of total)	6.6 (1.3)	6.6 (0.9)	
CON 18:2n6c (% of total)	33.6 (5.0)	34.5 (4.1)	F=0.250 p=0.78
BR-EX 18:2n6c (% of total)	37.2 (2.2)	37.7 (2.5)	
EX-BR 18:2n6c (% of total)	29.2 (3.3)	30.5 (0.8)	

Table 6 (con) Skeletal muscle phospholipid composition in a control (CON; *n*=6), breakfast-exercise (BR-EX; *n*=8) and exercise-breakfast (EX-BR; *n*=5) groups.

	Pre-intervention	Post-intervention	*time x group interaction*
CON C18n3 (% of total)	0.23 (0.06)	0.21 (0.05)	F=1.842 p=0.19
BR-EX C18n3 (% of total)	0.25 (0.05)	0.29 (0.04)	
EX-BR C18n3 (% of total)	0.22 (0.03)	0.26 (0.02)	
CON 20:4n6 (% of total)	14.4 (0.8)	15.0 (1.0)	F=1.862 p=0.19
BR-EX 20:4n6 (% of total)	14.9 (1.8)	14.0 (1.4)	
EX-BR 20:4n6 (% of total)	11.9 (1.3)	11.0 (1.7)	
CON 20:5n3 (% of total)	0.66 (0.21)	0.71 (0.18)	F=0.245 p=0.79
BR-EX 20:5n3 (% of total)	0.71 (0.12)	0.73 (0.12)	
EX-BR 20:5n3 (% of total)	0.64 (0.18)	0.69 (0.18)	
CON 20:0 (% of total)	0.13 (0.05)	0.09 (0.02)	F=1.141 p=0.34
BR-EX 20:0 (% of total)	0.08 (0.02)	0.08 (0.04)	
EX-BR 20:0 (% of total)	0.10 (0.04)	0.10 (0.07)	
CON 22:6n3 (% of total)	1.5 (0.4)	1.6 (0.5)	F=0.077 p=0.93
BR-EX 22:6n3 (% of total)	1.6 (0.2)	1.6 (0.2)	
EX-BR 22:6n3 (% of total)	1.3 (0.5)	1.4 (0.3)	
CON 24:1 (% of total)	0.13 (0.08)	0.13 (0.05)	F=0.021 p=0.98
BR-EX 24:1 (% of total)	0.10 (0.03)	0.10 (0.04)	
EX-BR 24:1 (% of total)	0.14 (0.09)	0.13 (0.08)	

Data are means and (SD). [a] denotes a difference in the change from pre-to post-intervention for CON versus EX-BR.

6441

Exercise training before nutrient provision augments intramuscular adaptations

Skeletal muscle AMPK protein levels increased ~3-fold with exercise training performed before- but not after-nutrient provision *versus* a no-exercise control group (**Figure 8A**). However, these increases did not translate into differential changes in proteins including CD36 and CPT-1 which are involved in fatty acid transport in skeletal muscle [both $p>0.05$; data available online (51)], or markers of mitochondrial oxidative capacity, including the protein levels of the OXPHOS complexes [all $p>0.05$; data available online (51)] or citrate synthase activity (change from baseline: -2.1 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ [-12.9 to 8.7] in CON, 7.6 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ [-1.2 to 16.4] in BR-EX and 6.5 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ [0.2 to 12.8] in EX-BR; $p>0.05$). There were also no differential changes in the content of insulin signaling proteins such as Akt2 or AS160 in response to nutrient-exercise timing ($p>0.05$; **Figure 8B**). However, there was a ~2-fold increase in skeletal muscle GLUT4 protein levels with exercise training performed before ($p=0.04$), but not after nutrient provision ($p=0.58$) *versus* a non-exercise control group (**Figure 8A**). There was also an increase in the protein levels of the CHC22 clathrin isoform and its associated adaptor protein (GGA2) relative to the CHC17 clathrin isoform, with exercise before *versus* after nutrient provision (both $p<0.05$; **Figure 8B**). When we examined the CHC22 isoform alone [data not shown but available online (51)]we noted baseline differences which may have confounded the interpretation of these fold-changes due to regression to the mean. We thus present the CHC22/CHC17 ratio (**Figure 8B**) to reflect GLUT4-associated clathrin-mediated membrane traffic relative to total clathrin-mediated membrane traffic.

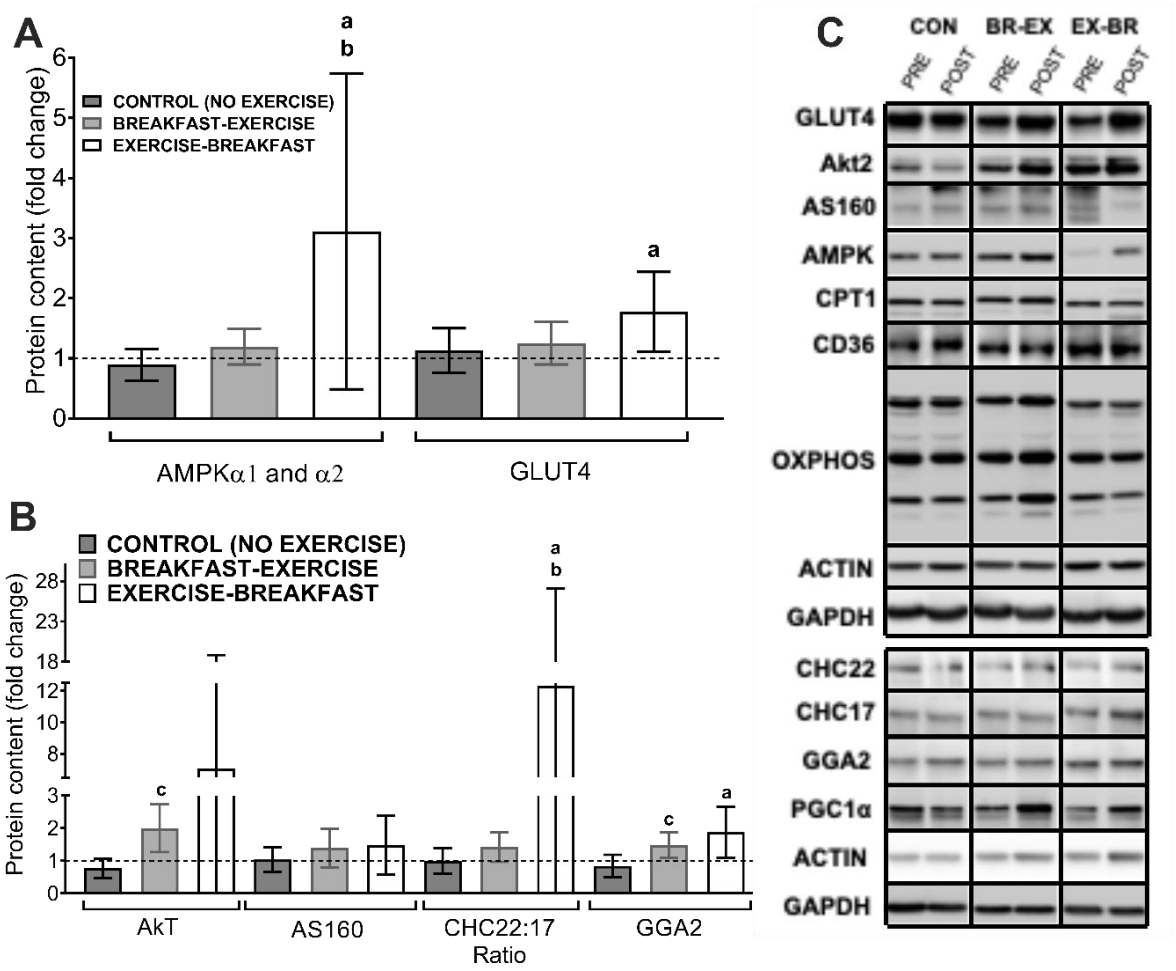

Figure 8. Pre-to-post intervention changes in the levels of energy-sensing proteins and proteins involved in insulin-sensitive GLUT4 trafficking in skeletal muscle (**A** and **B**). Representative immunoblots are shown (**C**) for each protein (including those reported in text but not shown in this figure) from the same participant as well as the loading controls used. All data are presented as means ± 95% CI and the dotted horizontal line represents the baseline (pre-intervention) values. For control n = 6, for breakfast-exercise n = 9 and for exercise-breakfast n = 5 men classified as overweight or obese. [a]difference between control *versus* exercise-breakfast; [b]difference between breakfast-exercise *versus* exercise-breakfast; [c]difference between control *versus* breakfast-exercise ($p < 0.05$).

DISCUSSION

This is the first study to investigate the effect of nutrient-exercise interactions on key aspects of metabolic health in people classified as overweight or obese. We found that a single exercise bout performed before, but not after, nutrient provision increased whole body and skeletal muscle lipid utilization. We then used a 6-week training program to reveal sustained, 2-fold increases in lipid utilization that were maintained throughout 6 weeks of exercise training performed before *versus* after nutrient provision. An oral glucose tolerance test-derived estimate of peripheral insulin sensitivity (the OGIS index) increased with exercise training before *versus* after nutrient provision and this was associated with increased lipid utilization during the exercise training intervention. Exercise training prior to nutrient provision also augmented remodeling of phospholipids and increased the levels of energy sensing (i.e. AMPK) and glucose transport proteins (i.e. GLUT4) in exercised skeletal muscle. These results indicate that nutrient-exercise timing modulates training responsiveness in overweight men and link lipid utilization during exercise to exercise-training-induced changes in aspects of metabolic health.

First, we showed that a single bout of exercise performed before *versus* after nutrient intake increased whole-body lipid utilization. A blunting of intramuscular triglyceride (IMTG) utilization has been shown in type I fibers of lean, healthy men in response to carbohydrate ingestion before and during exercise, compared to exercise in the fasted-state (22). Here, we demonstrated for the first time that exercise before *versus* after breakfast consumption increases net IMTG utilization in men classified as overweight or obese. Whilst the authors do acknowledge that absolute IMTG content may have been underestimated due to the analytical procedures used to estimate IMTG (i.e. use of Triton-X100 detergent, overnight drying of mounting medium), all samples were treated consistently. We also showed that net skeletal muscle glycogen utilization and acute skeletal muscle mRNA responses were largely unaffected by the same exercise performed before *versus* after breakfast. This is important, because muscle glycogen availability can alter muscle adaptations to training (27). Lower

muscle glycogen concentrations are therefore unlikely to have driven the training responses we observed in the **training study** with the present method of nutrient-exercise timing.

Altering substrate availability can also drive adaptive responses to exercise partly by modulating acute mRNA expression in exercised skeletal muscle (52). However, in the present study, only one measured gene was differentially expressed in response to exercise before *versus* after nutrient provision. Specifically, we observed less of an exercise-induced increase in skeletal muscle *PPARδ* expression with exercise before *versus* after nutrient provision, which is surprising given that *PPARδ* has been implicated in adaptations relating to oxidative capacity and lipid utilization (53). However, previous research has also shown no differential increase in *PPARδ* expression in skeletal muscle when exercise was performed with carbohydrate consumption before and during exercise *versus* in the fasted state (54). The different response observed in the present study might be because we assessed the effect of nutrient-exercise timing (nutrient provision before *versus* after exercise) rather than the omission *versus* ingestion of nutrients. This suggests that inferences cannot necessarily be extrapolated from studies assessing the effects of nutrient ingestion *versus* nutrient omission, to inform responses to models of nutrient-exercise *timing*.

In the **training study**, we then showed that acute increases in whole-body lipid utilization during a single bout of exercise performed before *versus* after nutrient intake were sustained throughout 6-weeks of exercise training. Moreover, only exercise training performed before nutrient intake reduced postprandial insulinemia and increased the oral glucose tolerance test-derived estimate of peripheral insulin sensitivity (i.e. the OGIS index). As the plasma C-peptide-to-insulin ratio was not differentially altered by nutrient-exercise timing, the reduction in postprandial insulinemia with exercise performed before *versus* after nutrient ingestion is likely to be due to a reduction in insulin secretion rather than an increase in hepatic insulin extraction (55). It should also be noted that difference between the exercise groups for the change in the OGIS index was also broadly

equivalent to the difference between individuals classified as having a healthy phenotype compared to individuals with impaired glucose tolerance (56).

Exercise training before *versus* after nutrient provision also resulted in augmented phospholipid remodeling in skeletal muscle. Moreover, the change in the saturated fatty acid content of skeletal muscle phospholipids with exercise correlated with the change in postprandial insulinemia. This supports prior observations that a higher proportion of saturated fatty acids in skeletal muscle phospholipids negatively correlates with insulin sensitivity (57). Single-leg exercise training has been used to show increased polyunsaturated fatty acid content of skeletal muscle phospholipids in an exercised *versus* non-exercised leg (58). Since that change was independent of dietary intake, the reduction in the saturated fatty content of phospholipids was likely due to a preferential upregulation of saturated fatty acid oxidation as a result of the higher energy expenditure (59,60). However, because this previous work involved changes in energy expenditure across experimental conditions, the role of lipid utilization independent of energy expenditure on phospholipid remodeling could not be explored. Here, we showed that skeletal muscle remodeling was increased with exercise performed before *versus* after nutrient provision, presumably due to increased lipid utilization in that condition.

AMPK is also nutrient sensitive and contributes to regulation of fatty acid utilization (61), mitochondrial biogenesis (62) and the expression of proteins involved in skeletal muscle glucose uptake, including GLUT4 and AS160 (63-65), which are key players in whole-body insulin sensitivity (66). We observed greater increases in the protein content of AMPK in skeletal muscle with exercise training before *versus* after nutrient intake. The increase in the GLUT4 content of skeletal muscle we observed with exercise before nutrient provision may be explained by this heightened AMPK response and, in turn, may have contributed to increases in the OGIS index following exercise training before *versus* after nutrient provision (67). Skeletal muscle AMPK can be activated by increased fatty acid availability, independent of muscle glycogen and AMP concentrations (68). Muscle glycogen

utilization can modulate *AMPK* and *GLUT4* mRNA expression with different exercise models (66). However, since we observed no difference in muscle glycogen utilization with altered nutrient-exercising timing in the **acute study**, the change in the GLUT4 content with exercise training before *versus* after breakfast is likely to be attributable to repeated increases in fatty acid availability, potentially through increases in the skeletal muscle AMPK content. The AMPK antibody we used detects both isoforms of the catalytic subunits of AMPK (AMPKα1 and α2). In human skeletal muscle, three different complexes have been described [α2β2γ1, α2β2γ3, and α1β2γ1; (69)] and our antibody therefore captured all complexes. Accordingly, we cannot speculate whether a specific heterotrimeric AMPK complex is predominately contributing to the increase in AMPK content that we report. As such, the effect of nutrient-exercise timing on AMPK activation warrants continued investigation.

The correct targeting and sequestration of GLUT4 into its intracellular insulin-responsive compartments is also important for insulin sensitivity in skeletal muscle (70,71). Clathrin heavy chain isoform 22 (CHC22) plays a specialized role in regulating GLUT4 sequestration in human skeletal muscle (72), protecting GLUT4 from degradation (73) and making it more available for insulin-stimulated release. We showed an increase in CHC22 protein levels in exercised muscle (relative to the exercise effects on CHC17 protein levels) with exercise before *versus* after nutrient provision. As the cognate clathrin CHC17 plays a widespread membrane traffic role in many tissues, CHC17 levels provide a benchmark for general membrane traffic changes compared to those in the GLUT4 pathway (74). The relative increase in CHC22 levels we observed thus suggests that exercise before nutrient provision not only augments GLUT4 protein levels, but potentially also the machinery necessary for the appropriate sequestration and targeting of GLUT4 to its insulin-responsive compartment. This may lead to improved GLUT4 translocation and contribute to the increases in the OGIS index we observed with exercise training before *versus* after nutrient intake. However, the CHC22 results reported here should be interpreted cautiously due to the relatively small sample size and variability in the individual CHC22

responses. Further work is therefore needed to investigate nutrient-exercise interactions and their effect on CHC22 levels. In addition, the remodeling of skeletal muscle phospholipids could have contributed to the ability of GLUT4 to fuse to the muscle-plasma membrane via less rigid arrays of phospholipid molecules in plasma membranes (75).

The greater increase in AMPK content we observed with exercise before nutrient provision did not further augment measured markers of mitochondrial biogenesis in skeletal muscle in response to exercise training in overweight men. This is in contrast to prior work demonstrating that carbohydrate ingestion before and during exercise suppresses exercise-induced increases in the content of proteins in skeletal muscle involved in fatty acid transport and oxidation (26). This further highlights that the model of nutrient-exercise timing that we employed (breakfast consumption before *versus* after exercise) might be distinct from other types of nutrient timing. Although changes in skeletal muscle mitochondrial content and/or oxidative capacity may be involved in regulating insulin sensitivity (76), the lack of differential response with exercise before *versus* after nutrition provision in this study suggests that these factors are unlikely to explain the changes in the OGIS index with the current model of nutrient-exercise timing employed (i.e. exercise before versus after breakfast). It is interesting that the intramuscular adaptations and changes in the OGIS index that we observed occurred in the presence of similar changes in body composition, self-reported daily dietary intake and total daily energy expenditure with altered nutrient-exercise timing. Notwithstanding other factors that may have contributed to the increases in oral glucose insulin sensitivity with exercise before *versus* after nutrient ingestion, this highlights lipid metabolism as a potentially important mechanism explaining the improvement in OGIS with regular exercise performed before *versus* after breakfast.

It should also be noted that the responses observed for OGIS were an interaction between groups, and thus the response to exercise before nutrient provision is an increase relative to the non-exercise control group and the exercise after nutrient intake group. Accordingly, these data may be specific to high-

carbohydrate provision and although this is typical of breakfasts in developed countries, it remains to be seen whether lower-carbohydrate meals produce similar effects. Potential limitations in our work also include the absence of a non-exercise fasting group, which would have allowed us to explore a role of extended morning fasting *per se* in the **training study**. However, our prior work has already shown that extended morning fasting in an absence of exercise may impair insulin sensitivity and increase postprandial insulinemia in obese humans (77).

To summarize, the present data are the first to show that exercise training before *versus* after carbohydrate (i.e. breakfast) consumption affects responsiveness to exercise training in men classified as overweight or obese, including greater remodeling of skeletal muscle phospholipids, adaptations of proteins involved in nutrient sensing and glucose transport in skeletal muscle, and increases in and index of oral glucose insulin sensitivity. These data suggest that exercising in a fasted state can augment the adaptive response to exercise, without the need to increase the volume, intensity, or perception of effort of exercise. These responses may be linked to the acute increases in lipid utilization during every bout of exercise performed in the fasted- *versus* the fed-state (a difference that is sustained throughout a period of training over 6-weeks). These findings therefore have implications for future research and clinical practice. For example, exercise training studies should account for nutrient-exercise timing if aspects of metabolic control are an outcome measure. Secondly, to increase lipid utilization and oral glucose insulin sensitivity with training, endurance-type exercise should be performed before *versus* after nutrient intake (i.e. in the fasted state).

REFERENCES FOR CHAPTER 6

1. DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *Journal of Clinical Investigation*. 1985;76(1):149-155.
2. Reaven GM. Role of insulin resistance in human disease. *Diabetes*. 1988;37(12):1595-1607.
3. Tricò D, Natali A, Arslanian S, Mari A, Ferrannini E. Identification, pathophysiology, and clinical implications of primary insulin hypersecretion in nondiabetic adults and adolescents. *JCI Insight*. 2018;3(24):Epub: doi: 10.1172/jci.insight.124912.
4. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. *JAMA*. 1999;282(16):1523-1529.
5. McLaughlin T, Lamendola C, Liu A, Abbasi F. Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. *The Journal of Clinical Endocrinology & Metabolism*. 2011;96(11):E1756-E1760.
6. Borghouts L, Keizer H. Exercise and insulin sensitivity: a review. *International Journal of Sports Medicine*. 2000;21(1):1-12.
7. Sylow L, Richter EA. Current advances in our understanding of exercise as medicine in metabolic disease. *Current Opinion in Physiology*. 2019;Epub: https://doi.org/10.1016/j.cophys.2019.04.008.
8. Lund S, Pryor PR, Ostergaard S, Schmitz O, Pedersen O, Holman GD. Evidence against protein kinase B as a mediator of contraction-induced glucose transport and GLUT4 translocation in rat skeletal muscle. *FEBS Lett*. 1998;425(3):472-474.
9. Geiger PC, Han DH, Wright DC, Holloszy JO. How muscle insulin sensitivity is regulated: testing of a hypothesis. *Am J Physiol Endocrinol Metab*. 2006;291(6):E1258-1263.
10. Hansen PA, Wang W, Marshall BA, Holloszy JO, Mueckler M. Dissociation of GLUT4 translocation and insulin-stimulated glucose transport in transgenic mice overexpressing GLUT1 in skeletal muscle. *J Biol Chem*. 1998;273(29):18173-18179.
11. Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *Journal of Applied Physiology*. 1984;56(4):831-838.
12. O'Gorman DJ, Karlsson HK, McQuaid S, Yousif O, Rahman Y, Gasparro D, Glund S, Chibalin AV, Zierath JR, Nolan JJ. Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. *Diabetologia*. 2006;49(12):2983-2992.
13. Andersson A, Sjödin A, Olsson R, Vessby B. Effects of physical exercise on phospholipid fatty acid composition in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*. 1998;274(3):432-438.

14. Helge JW, Dela F. Effect of training on muscle triacylglycerol and structural lipids: a relation to insulin sensitivity? *Diabetes*. 2003;52(8):1881-1887.
15. de Lannoy L, Clarke J, Stotz PJ, Ross R. Effects of intensity and amount of exercise on measures of insulin and glucose: Analysis of inter-individual variability. *PloS one*. 2017;12(5):e0177095.
16. Atkinson G, Batterham AM. True and false interindividual differences in the physiological response to an intervention. *Experimental physiology*. 2015;100(6):577-588.
17. Chen Y-C, Travers RL, Walhin J-P, Gonzalez JT, Koumanov F, Betts JA, Thompson D. Feeding influences adipose tissue responses to exercise in overweight men. *American Journal of Physiology-Endocrinology and Metabolism*. 2017;313(1):84-93.
18. Edinburgh RM, Hengist A, Smith HA, Travers RL, Koumanov F, Betts JA, Thompson D, Walhin J-P, Wallis GA, Hamilton DL, Stevenson EJ, Tipton KD, Gonzalez J. Pre-Exercise Breakfast Ingestion versus Extended Overnight Fasting Increases Postprandial Glucose Flux after Exercise in Healthy Men. *American Journal of Physiology-Endocrinology and Metabolism*. 2018;315(5):1062-1074.
19. Gonzalez JT, Veasey RC, Rumbold PL, Stevenson EJ. Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. *British Journal of Nutrition*. 2013;110(4):721-732.
20. Wallis GA, Gonzalez JT. Is exercise best served on an empty stomach? *Proc Nutr Soc*. 2019;78(1):110-117.
21. Edinburgh RM, Hengist A, Smith HA, Travers RL, Koumanov F, Betts JA, Thompson D, Walhin JP, Wallis GA, Hamilton DL, Stevenson EJ, Tipton KD, Gonzalez JT. Pre-exercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. 2018;Accepted-ahead-of-print.
22. De Bock K, Richter EA, Russell A, Eijnde BO, Derave W, Ramaekers M, Koninckx E, Leger B, Verhaeghe J, Hespel P. Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. *The Journal of Physiology*. 2005;564(2):649-660.
23. Cluberton LJ, McGee SL, Murphy RM, Hargreaves M. Effect of carbohydrate ingestion on exercise-induced alterations in metabolic gene expression. *Journal of Applied Physiology*. 2005;99(4):1359-1363.
24. Civitarese AE, Hesselink MK, Russell AP, Ravussin E, Schrauwen P. Glucose ingestion during exercise blunts exercise-induced gene expression of skeletal muscle fat oxidative genes. *American Journal of Physiology-Endocrinology and Metabolism*. 2005;289(6):1023-1029.
25. Stocks B, Dent JR, Ogden HB, Zemp M, Philp A. Postexercise skeletal muscle signaling responses to moderate-to high-intensity steady-state exercise in the fed or fasted state. *American Journal of Physiology-Endocrinology and Metabolism*. 2018;316(2):230-238.
26. Van Proeyen K, Szlufcik K, Nielens H, Pelgrim K, Deldicque L, Hesselink M, Van Veldhoven PP, Hespel P. Training in the fasted state improves

glucose tolerance during fat-rich diet. *The Journal of Physiology*. 2010;588(21):4289-4302.
27. Burke LM, Hawley JA. Swifter, higher, stronger: What's on the menu? *Science*. 2018;362(6416):781-787.
28. Gonzalez JT, Richardson JD, Chowdhury EA, Koumanov F, Holman GD, Cooper S, Thompson D, Tsintzas K, Betts JA. Molecular adaptations of adipose tissue to 6 weeks of morning fasting vs. daily breakfast consumption in lean and obese adults. *The Journal of Physiology*. 2018;596(4):609-622.
29. Wallis GA, Gonzalez JT. Is exercise best served on an empty stomach? *Proceedings of the Nutrition Society*. 2018;78(1):110-117.
30. FAO. Human energy requirements. Report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17-24 October 2001. http://wwwfaoorg/3/y5686e/y5686e07htm#bm073.
31. Borg G. Perceived exertion: a note on 'history' and methods. *Medicine and Science in Sports and Exercise*. 1973;5(2):90-93.
32. Brouns F, Bjorck I, Frayn K, Gibbs A, Lang V, Slama G, Wolever T. Glycaemic index methodology. *Nutrition Research Reviews*. 2005;18(1):145-171.
33. Gonzalez JT, Fuchs CJ, Smith FE, Thelwall PE, Taylor R, Stevenson EJ, Trenell MI, Cermak NM, Van Loon LJ. Ingestion of glucose or sucrose prevents liver but not muscle glycogen depletion during prolonged endurance-type exercise in trained cyclists. *American Journal of Physiology-Endocrinology and Metabolism*. 2015;309(12):1032-1039.
34. Fletcher G, Eves FF, Glover EI, Robinson SL, Vernooij CA, Thompson JL, Wallis GA. Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *Am J Clin Nutr*. 2017;105(4):864-872.
35. Edinburgh R, Hengist A, Smith HA, Betts JA, Thompson D, Walhin J-P, Gonzalez JT. Prior exercise alters the difference between arterialised and venous glycaemia: implications for blood sampling procedures. *British Journal of Nutrition*. 2017;117(10):1414-1421.
36. Passonneau J, Lauderdale V. A comparison of three methods of glycogen measurement in tissues. *Analytical Biochemistry*. 1974;60(2):405-412.
37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*. 2001;25(4):402-408.
38. Satoh S, Nishimura H, Clark AE, Kozka IJ, Vannucci SJ, Simpson IA, Quon MJ, Cushman SW, Holman GD. Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *Journal of Biological Chemistry*. 1993;268(24):17820-17829.
39. Hoshino S, Sakamoto K, Vassilopoulos S, Camus SM, Griffin CA, Esk C, Torres JA, Ohkoshi N, Ishii A, Tamaoka A. The CHC22 clathrin-GLUT4 transport pathway contributes to skeletal muscle regeneration. *PloS one*. 2013;8(10):e77787.

40. Näthke IS, Heuser J, Lupas A, Stock J, Turck CW, Brodsky FM. Folding and trimerization of clathrin subunits at the triskelion hub. *Cell*. 1992;68(5):899-910.
41. Kjøbsted R, Hingst JR, Fentz J, Foretz M, Sanz MN, Pehmøller C, Shum M, Marette A, Mounier R, Treebak JT, Wojtaszewski JFP, Viollet B, Lantier L. AMPK in skeletal muscle function and metabolism. *FASEB J*. 2018;32(4):1741-1777.
42. Miklosz A, Chabowski A, Zendzian-Piotrowska M, Gorski J. Effects of hyperthyroidism on lipid content and composition in oxidative and glycolytic muscles in rats. *J Physiol Pharmacol*. 2012;63:403-410.
43. Mikłosz A, Łukaszuk B, Żendzian-Piotrowska M, Kurek K, Chabowski A. The effects of AS160 modulation on fatty acid transporters expression and lipid profile in L6 myotubes. *Cellular Physiology and Biochemistry*. 2016;38(1):267-282.
44. Compher C, Frankenfield D, Keim N, Roth-Yousey L, Group EAW. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *Journal of the American Dietetic Association*. 2006;106(6):881-903.
45. Frayn K. Calculation of substrate oxidation rates in vivo from gaseous exchange. *Journal of Applied Physiology*. 1983;55(2):628-634.
46. Jeukendrup A, Wallis G. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *International Journal of Sports Medicine*. 2005;26(1):28-37.
47. Thompson D, Batterham AM, Bock S, Robson C, Stokes K. Assessment of low-to-moderate intensity physical activity thermogenesis in young adults using synchronized heart rate and accelerometry with branched-equation modeling. *The Journal of Nutrition*. 2006;136(4):1037-1042.
48. Villars C, Bergouignan A, Dugas J, Antoun E, Schoeller DA, Roth H, Maingon A-C, Lefai E, Blanc S, Simon C. Validity of combining heart rate and uniaxial acceleration to measure free-living physical activity energy expenditure in young men. *Journal of applied physiology*. 2012;113(11):1763-1771.
49. Brage S, Westgate K, Franks PW, Stegle O, Wright A, Ekelund U, Wareham NJ. Estimation of free-living energy expenditure by heart rate and movement sensing: a doubly-labelled water study. *PloS one*. 2015;10(9):Epub: https://doi.org/10.1371/journal.pone.0137206.
50. Mari A, Pacini G, Brazzale AR, Ahrén B. Comparative evaluation of simple insulin sensitivity methods based on the oral glucose tolerance test. *Diabetologia*. 2005;48(4):748-751.
51. Edinburgh R, Bradley H, Abdullah N, Robinson S, Chrzanowski-Smith O, Walhin J, Joanisse S, Manolopoulos K, Philp A, Hengist A, Chabowski A, Brodsky F, Koumanov F, Betts J, Thompson D, Wallis G, Gonzalez J. Dataset for "Lipid metabolism links nutrient-exercise timing to insulin sensitivity in overweight men". *University of Bath Research Data Archive* https://doiorg/1015125/BATH-00672. 2019.
52. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional

and mitochondrial proteins during training in human skeletal muscle. *The Journal of Physiology*. 2010;588(23):4795-4810.
53. Ehrenborg E, Krook A. Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor δ. *Pharmacological Reviews*. 2009;61(3):373-393.
54. Russell AP, Hesselink MK, Lo SK, Schrauwen P. Regulation of metabolic transcriptional co-activators and transcription factors with acute exercise. *The FASEB journal*. 2005;19(8):986-988.
55. Polonsky KS, Rubenstein AH. C-peptide as a measure of the secretion and hepatic extraction of insulin: pitfalls and limitations. *Diabetes*. 1984;33(5):486-494.
56. Mari A, Pacini G, Murphy E, Ludvik B, Nolan JJ. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. *Diabetes Care*. 2001;24(3):539-548.
57. Vessby B, Tengblad S, Lithell H. Insulin sensitivity is related to the fatty acid composition of serum lipids and skeletal muscle phospholipids in 70-year-old men. *Diabetologia*. 1994;37(10):1044-1050.
58. Helge JW, Wu BJ, Willer M, Daugaard JR, Storlien LH, Kiens B. Training affects muscle phospholipid fatty acid composition in humans. *Journal of Applied Physiology*. 2001;90(2):670-677.
59. Bergouignan A, Trudel G, Simon C, Chopard A, Schoeller DA, Momken I, Votruba SB, Desage M, Burdge GC, Gauquelin-Koch G. Physical inactivity differentially alters dietary oleate and palmitate trafficking. *Diabetes*. 2009;58(2):367-376.
60. Lefai E, Blanc S, Momken I, Antoun E, Chery I, Zahariev A, Gabert L, Bergouignan A, Simon C. Exercise training improves fat metabolism independent of total energy expenditure in sedentary overweight men, but does not restore lean metabolic phenotype. *International Journal Of Obesity*. 2017;41(1):1728-1736.
61. Handschin C, Spiegelman BM. Peroxisome Proliferator-Activated Receptor γ Coactivator 1 Coactivators, Energy Homeostasis, and Metabolism. *Endocrine Reviews*. 2006;27(7):728-735.
62. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*. 1999;98(1):115-124.
63. Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, Holloszy JO. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca2+. *American Journal of Physiology-Endocrinology and Metabolism*. 2002;282(5):1008-1013.
64. Frøsig C, Jørgensen SB, Hardie DG, Richter EA, Wojtaszewski JF. 5′-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*. 2004;286(3):411-417.
65. Friedrichsen M, Mortensen B, Pehmøller C, Birk JB, Wojtaszewski JF. Exercise-induced AMPK activity in skeletal muscle: role in glucose

uptake and insulin sensitivity. *Molecular and Cellular Endocrinology*. 2013;366(2):204-214.
66. Richter EA, Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological Reviews*. 2013;93(3):993-1017.
67. Leturque A, Loizeau M, Vaulont S, Salminen M, Girard J. Improvement of Insulin Action in Diabetic Transgenic Mice Selectively Overexpressing GLUT4 in Skeletal Muscle. *Diabetes*. 1996;45(1):23-27.
68. Watt MJ, Steinberg GR, Chen ZP, Kemp BE, Febbraio MA. Fatty acids stimulate AMPK-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. *Journal of Physiology*. 2006;574(1):139-147.
69. Wojtaszewski JF, Birk JB, Frøsig C, Holten M, Pilegaard H, Dela F. 5'AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. *Journal of Physiology*. 2005;564:563-573.
70. Hansen PA, Nolte LA, Chen MM, Holloszy JO. Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *Journal of Applied Physiology*. 1998;85(4):1218-1222.
71. Fisher JS, Gao J, Han D-H, Holloszy JO, Nolte LA. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *American Journal of Physiology-Endocrinology and Metabolism*. 2002;282(1):E18-E23.
72. Vassilopoulos S, Esk C, Hoshino S, Funke BH, Chen C-Y, Plocik AM, Wright WE, Kucherlapati R, Brodsky FM. A role for the CHC22 clathrin heavy-chain isoform in human glucose metabolism. *Science*. 2009;324(5931):1192-1196.
73. Fumagalli M, Camus SM, Diekmann Y, Burke A, Camus MD, Norman PJ, Joseph AP, Abi-Rache L, Benazzo A, Rasteiro R, Mathieson I, Topf M, Parham P, Thomas MG, Brodsky FM. Genetic diversity of CHC22 clathrin impacts its function in glucose metabolism. *eLife*. 2019;8.
74. Dannhauser PN, Camus SM, Sakamoto K, Sadacca LA, Torres JA, Camus MD, Briant K, Vassilopoulos S, Rothnie A, Smith CJ, Brodsky FM. CHC22 and CHC17 clathrins have distinct biochemical properties and display differential regulation and function. *The Journal of Biological Chemistry*. 2017;292(51):20834-20844.
75. Weijers R. Lipid composition of cell membranes and its relevance in type 2 diabetes mellitus. *Current diabetes reviews*. 2012;8(5):390-400.
76. Apostolopoulou M, Strassburger K, Herder C, Knebel B, Kotzka J, Szendroedi J, Roden M. Metabolic flexibility and oxidative capacity independently associate with insulin sensitivity in individuals with newly diagnosed type 2 diabetes. *Diabetologia*. 2016;59(10):2203-2207.
77. Chowdhury EA, Richardson JD, Holman GD, Tsintzas K, Thompson D, Betts JA. The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults. *The American Journal of Clinical Nutrition*. 2016;103(3):747-756.

CHAPTER 7 - GENERAL DISCUSSION

The aim of this thesis was to characterise the acute and training responses to exercise performed during morning fasting (with breakfast omission or breakfast consumption post-exercise) compared to exercise performed after consuming breakfast, with a specific focus on substrate metabolism, postprandial glycemia, intramuscular adaptations and energy balance. This first involved an application of glucose tracer methods in combination with muscle sampling to investigate postprandial blood glucose flux after a single bout of exercise. Consuming *versus* omitting breakfast before a single bout of exercise increased plasma glucose flux after a post-exercise meal, despite blood glucose concentrations not substantially differing in those conditions. It was then shown that an energy deficit from omitting breakfast before exercise is not compensated for over a 24 h period via either an increase in energy intake or a decrease in energy expenditure in healthy men. Finally, if the *acute* metabolic- and intramuscular-responses to altered nutrient-exercise timing translates into *enduring adaptations* with a repeated exposure to these stimuli in overweight men was investigated. In that study, participants were prescribed the same breakfast and training over 6 weeks, with the only difference between the exercise groups being in the timing of carbohydrate consumption in relation to exercise. In six weeks, exercise training before *versus* after nutrient-intake increased oral glucose insulin sensitivity and augmented key adaptive responses to exercise in skeletal muscle. Therefore, for men classified as overweight or obese, it is preferable to perform exercise sessions before *versus* after consuming carbohydrates to heighten the metabolic benefits of training. This finding could have important implications for future exercise recommendations.

The acute and training study in this thesis had some differences, namely in the population studied and if the main comparison was breakfast consumption *versus* omission or altered breakfast timing (pre- *versus* post-exercise). Despite this, a consistent finding was that glycaemic responses to post-exercise meals depends on if breakfast is consumed before exercise. A previous study showed that when a single exercise bout is performed after breakfast, blood glucose concentrations

were increased after a post-exercise meal *versus* breakfast consumption at rest, but when breakfast was omitted before exercise this response was less apparent *versus* breakfast omission and rest (15). Using glucose tracer methods in **Chapter 3**, it was shown that plasma glucose flux is increased at post-exercise meals with prior breakfast consumption *versus* omission, but clear differences in blood glucose concentrations were not apparent in that chapter. One difference between the research in this thesis and the previous work was in the sampling of arterialised blood *versus* venous blood respectively, which alters the measured concentrations of blood glucose (428). The magnitude of any arterialised-venous differences also depends on the net uptake or release of metabolites from tissue beds in proximity to the blood sampling site. This was studied in **Chapter 4**, where performing prior exercise was shown to reduce postprandial arterialised-venous differences for blood glucose concentrations compared to rest, which may explain why a higher blood glucose response was shown with exercise *versus* rest with venous blood (15), but not in **Chapter 3**, when arterialised blood was sampled.

In **Chapter 3**, the increase in plasma glucose appearance rates with breakfast consumption before exercise was primarily attributable to glucose from the post-exercise oral glucose tolerance test (OGTT). However, systemic intestinal fatty acid binding protein concentrations, as a marker of intestinal damage, were lower if breakfast was consumed *versus* omitted before exercise. That finding suggests that exercise-induced intestinal damage was lessened in that trial and a better maintenance of splanchnic perfusion during exercise with prior feeding is a likely explanation (10). If splanchnic perfusion was better maintained during exercise with prior feeding, this may have also facilitated the higher OGTT-derived plasma glucose appearance compared to when breakfast was omitted before exercise, which should now be investigated. A higher postprandial plasma glucose disposal rate was also shown with breakfast consumption *versus* omission pre-exercise, despite postprandial insulinemia being reduced. Intramuscular insulin-signaling pathways mediate blood glucose disposal in response to exercise/nutrition (492). However, Akt activation at Thr^{308} and Ser^{473} and $AS160^{Thr642}$ phosphorylation were not clearly different at 2-h post-OGTT with breakfast consumption *versus*

omission before exercise, suggesting that differences in the activation of proteins in the early stages of intramuscular insulin signalling were not responsible for the higher plasma glucose disposal rates if breakfast was consumed before exercise.

However, heightened intramuscular AMPK signalling was apparent after exercise with prior breakfast consumption *versus* omission, which may have contributed to the higher blood glucose disposal rates in that trial (493). The ingestion of large amounts (> 200 g) of carbohydrate before *and* during exercise can blunt AMPK signaling in muscle (315, 494). However, when a carbohydrate dose (< 120 g) more representative of a breakfast consumed in developed countries (186) is consumed pre-exercise, net muscle glycogen utilisation during the exercise might be increased, especially when no carbohydrate is ingested during the exercise (293). This may be because exercise after a high-glycaemic index, carbohydrate-rich breakfast can facilitate lower blood glucose concentrations in the early stage of the activity (273). A breakfast-induced increase in insulin concentrations may stimulate carbohydrate utilisation and reduce net NEFA mobilisation from the adipose-tissue. Then if exercise is started soon after, the availability of energy-deriving substrates in the blood may not be sufficient to meet the energy demands of the activity, with a combination of these stimuli increasing muscle glycogen utilisation, and in turn, the exercise-induced AMPK response (313).

There is also evidence that pre-exercise muscle glycogen concentrations would not be higher with breakfast consumption *versus* omission before exercise, especially if the activity was commenced < 2 h after the meal. A series of studies performed by Taylor *et al.* used ^{13}C nuclear magnetic resonance spectroscopy to show the slow time course for muscle-glycogen synthesis after consumption of a mixed-macronutrient and ~ 60 % carbohydrate breakfast. In the first of these studies (495) muscle glycogen concentrations initially decreased and then did not start to rise above basal concentrations until ~ 2 h post-meal, with the increase above basal concentrations not reaching significance until ~ 3 h post-meal. A second study then confirmed this observation in healthy men and also showed blunted muscle-glycogen synthesis in humans with T2D (496). It was inferred that

the predominant fate of the glucose taken up by skeletal muscle in the immediate (< 2 h) post-breakfast period is oxidation, before a switch towards net glycogen synthesis thereafter. This suggests that muscle glycogen concentrations may not be higher prior to exercise performed < 2 h after consuming breakfast *versus* breakfast omission. Thus, if muscle glycogen utilisation is indeed greater during post-breakfast exercise (if no carbohydrate is ingested during exercise) muscle glycogen concentrations could be lower after the activity is completed. In light of the data in **Chapter 3**, muscle glycogen concentrations and AMPK signalling with exercise after breakfast consumption *versus* omission should now be explored.

In **Chapter 3** it was shown that substrate metabolism during- and intramuscular signalling after *a single bout* of exercise performed during fasting can be altered by consuming a prior breakfast. Adaptations relating to postprandial metabolism after *exercise training* may also depend on nutrient timing in relation to exercise (8). If apparent, this may be attributable to higher rates of IMTG and/or adipose-tissue derived plasma NEFA utilisation during every bout of exercise performed before *versus* after nutrient intake, and/or increases in the content and activity of proteins involved in lipid metabolism after training (12, 13, 308). This is because defects related to lipid metabolism and/or a low turnover of endogenous lipid pools are likely to be apparent in obesity and have been associated with insulin resistance (9). The only prior training study which investigated whole-body insulin sensitivity under conditions of altered exercise-nutrient timing, suggested that feeding carbohydrates before and during-exercise blunted the increases in oral glucose insulin sensitivity compared to a non-exercise group, that was apparent when exercise was performed before any carbohydrate was consumed (11). However, that study was completed in healthy men during hyper-caloric, high-fat feeding and so those results are not easily applied to obesity or situations most representative of daily living in developed countries (186).

In **Chapter 6**, it was shown that when men classified as overweight or obese start exercise training, performing exercise sessions before *versus* after carbohydrate-ingestion increased oral glucose insulin sensitivity. This occurred despite no

measurable differences between the groups for changes in oxidative capacity at a whole-body level ($\dot{V}O_2$ peak) or in skeletal muscle, changes in the capacity for lipid utilisation at a whole-body level (peak fat utilisation rates during exercise) or in skeletal muscle (the content of CPT-1 and CD36) or changes in body-mass, self-reported energy intake or energy expenditure. However, a positive relationship between the changes in oral glucose insulin sensitivity and cumulative whole-body lipid utilisation during training was apparent, highlighting a possible link between the turnover of endogenous lipid pools and insulin sensitivity. In men classified as overweight or obese, consuming breakfast before *versus* after exercise decreases blood NEFA and glycerol concentrations (suggestive of a suppression of lipolysis in the adipose-tissue) during exercise and blunts the utilisation of IMTG (as shown in **Chapter 6**). A higher turnover of lipids from the adipose tissue has been associated with better metabolic health (167, 168) and a regular utilisation of IMTG is associated with increased insulin sensitivity, likely by alleviating any accumulation of lipid metabolites that interfere with insulin signalling (9). This supports the correlation between cumulative lipid utilisation and insulin sensitivity shown in **Chapter 6**, and suggests that for insulin sensitivity, a regular turnover of endogenous lipid stores may be important.

The phospholipid (PL) content of skeletal muscle may be a determinant of insulin sensitivity (134). Previous research has shown that exercise training can result in remodelling of the PL pool (325) likely due to an upregulation of saturated fatty acid oxidation with a higher energy expenditure (326). However, as all prior work has involved changes in energy expenditure a role of lipid utilisation independent of energy expenditure in PL remodelling was unclear. In **Chapter 6**, exercising before *versus* after breakfast was a greater stimulus for PL remodelling potentially due to increased lipid utilisation (326) and/or an increased accumulation of energy sensing proteins such as AMPK or PGC1α in skeletal muscle (327). In addition, the GLUT4 content in the exercised muscle was increased to a greater extent with exercise before, but not after breakfast *versus* a no-exercise group. This is consistent with the increased GLUT4 protein content after exercise training during morning fasting, but not if carbohydrate is fed before- and during-

exercise in healthy men (265). Eating breakfast before exercise may therefore blunt adaptive responses relating to energy sensing in muscle, which could in turn have implications for both PL remodelling (327) and the GLUT4 response to the training (165). The apparent disparity between the acute and training studies in this thesis for the post-exercise AMPK response suggests that acute responses to exercise do not always translate into enduring training adaptations and/or that responses to altered nutrient-exercise timing may be specific to the model that is employed. In **Chapter 6**, an increase in the CHC22 protein levels in skeletal muscle with exercise before *versus* after breakfast was also detected. CHC22 has a role in forming insulin-responsive GLUT4 vesicles and more research in humans is now needed to clarify the role of nutrient-exercise timing for CHC22. Collectively, the results in **Chapter 6** showed that exercise training before *versus* after nutrient-intake may be more efficacious for increasing oral glucose insulin sensitivity in overweight humans and that this response may be linked to increased lipid utilisation and augmented intramuscular adaptations (**Figure 7.1**).

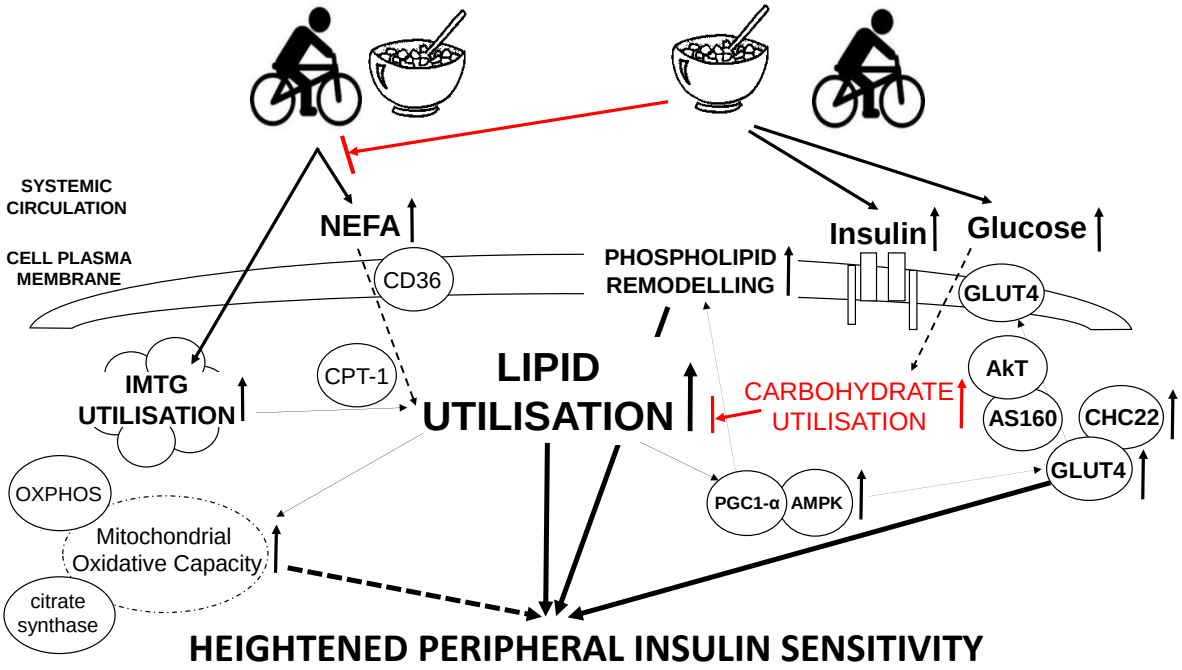

Figure 7.1. Mechanisms by which exercising before *versus* after nutrient intake may increase oral glucose tolerance test derived estimates of peripheral insulin sensitivity in humans classified as overweight or obese. Over six weeks, exercise performed before (*versus* after) breakfast resulted in increased lipid utilisation during each exercise session (increased cumulative lipid utilisation), an increased content of proteins involved in GLUT4 trafficking and insulin signalling and remodelling of the phospholipid pool in exercised skeletal muscle. Markers of mitochondrial oxidative capacity and capacity for lipid utilisation (CPT-1 or CD36 protein levels) did not clearly differ in skeletal muscle or when measured at a whole-body level (i.e. peak oxygen uptake and fat utilisation rates during an incremental exercise test) with exercise before *versus* after nutrient ingestion. In this figure the dashed line shows that markers of mitochondrial oxidative capacity were not differentially altered with nutrient-exercise timing despite oral glucose insulin sensitivity increasing with exercise training before *versus* after breakfast.

Abbreviations:
GLUT4 = glucose transporter 4
NEFA = non-esterified fatty acid
IMTG = intramuscular triglyceride
AMPK = 5' AMP-activated protein kinase
PGC1α = peroxisome proliferator activated receptor gamma coactivator 1 alpha
OXPHOS = oxidative phosphorylation complexes
CPT-1 = carnitine palmitoyltransferase I
FAT/CD36 = fatty acid translocase/cluster of differentiation 36
Akt = protein kinase B;
AS160 = Akt substrate of 160 kDa
CHC22 = clathrin heavy-chain isoform 22

A second aim of this thesis was to investigate changes in energy balance with altered nutrient-exercise timing. Prior research demonstrated that the omission of breakfast before exercise was incompletely compensated for via a higher post-exercise energy intake in healthy men (15, 16). A lower energy intake over 24 h with pre-exercise breakfast omission has also been attributed to a lower energy intake from post-exercise meals and snacks (16). This may be explained by the glycogenostatic theory, which speculates that reducing carbohydrate utilisation during exercise may help to protect against compensation through a higher post-exercise energy intake (17). The research in **Chapter 5** showed that in healthy men, the omission *versus* consumption of breakfast before exercise was not compensated for with a higher post-exercise energy intake, nor a decreased energy expenditure, creating a more negative 24-h energy balance. Men with higher blood glucose disposal rates during fasted-exercise (suggesting a higher rate of liver carbohydrate utilisation) also consumed more energy at lunch *versus* their resting trial. This is consistent with the evidence that a higher liver glycogen content in mice is associated with a lower energy intake and together suggests that the liver glycogen content may be a regulator of post-exercise energy intake compensation (355, 469). However, this finding needs to be confirmed with direct measures of liver glycogen utilisation during exercise. The implications of these findings and this research area was recently reviewed by Dr Mark Hopkins (497).

Due to the incomplete compensation for breakfast omission prior to exercise, this strategy might create the conditions for greater losses in body mass compared to eating breakfast before exercise. However, any acute change in energy balance over 24 h needs to persist over a prolonged time period for changes in body mass (251). As adjustments in energy intake to energy deficits from exercise are thought to be regulated over > 24 h (498, 499), more research is now needed to investigate long-termer changes in energy balance and body mass with regular exercise training and breakfast omission *versus* consumption. However, regularly exercising in a fasted state and consuming breakfast after the exercise does not appear to increase losses in body- or fat-mass over 4-6 weeks, relative to when the same food is consumed pre- and/or during exercise (8). The research in

Chapter 6 was the first to assess changes in all components of energy balance when manipulating nutrient-exercise timing during supervised exercise training in men classified as overweight. Performing exercise increased average daily PAEE compared to a non-exercise group, but no meaningful differences in average daily self-reported energy intake, objectively measured energy expenditure, nor changes in body mass or the ratio of waist-to-hip circumference were evident between the two exercise groups. This suggests that exercising before *versus* after breakfast might not create the conditions for greater losses in body mass when the same nutrition is consumed post-exercise. Future research could now establish whether the acute energy deficit created by omitting breakfast and exercising translates into greater body mass losses over time.

One potential concern with exercise during morning fasting is a detrimental effect this could have for whole-body protein balance. Protein balance is determined by rates of protein synthesis *versus* protein breakdown, with synthesis exceeding breakdown following meals and the converse of this apparent during periods of fasting, so that a balance is achieved over a typical day (500). After an overnight fast the reduced availability of dietary amino acids and low circulating insulin concentrations shifts this equilibrium towards protein breakdown (501). Fasting for 72 h doubles rates of amino acid efflux from muscle *versus* a 10-h fast and liberated amino acids can undergo gluconeogenesis, thus allowing protein to contribute to energy metabolism (502). Whilst a 72-h fast is not representative of daily living for most people in developed countries, exercise during fasting *versus* after nutrient intake may alter protein balance, as fasting and exercise depress muscle protein synthesis, with a positive protein balance only achieved if amino acids availability is increased (503). In **Chapter 3**, a more negative 24-h protein balance was shown with breakfast omission *versus* consumption before exercise (mean [n95% CI]: 25 g [18 to 32] *versus* 35 g [25 to 44]; p=0.01) which is likely due to an incomplete compensation in energy intake for the 19 g of protein in the breakfast. The effect of only altering nutrient-exercise *timing* on protein balance during exercise training should be studied, as ingesting protein in isolation before *versus* after exercise has a similar effect on muscle protein synthesis (504).

As a higher turnover of endogenous lipid pools with exercise performed before *versus* after nutrient intake might, at least partially, explain augmented health-related adaptations to training (167, 168), understanding any effect of protein ingestion on lipid utilisation during exercise is important. Including whey protein in meals (50 % protein, 40 % carbohydrate and 10 % fat) consumed during rest does not suppress rates of lipid utilisation to the same extent as when an isoenergetic carbohydrate-only meal is consumed, even in the presence of blood insulin concentrations that do not clearly differ (505). In another cross-over study, 10 men performed 45 min of moderate-intensity cycling after prior muscle glycogen depletion (506). They ingested 20 g of casein hydrolysate 45 min before-, 10 g during- and 20 g post-exercise or a placebo. No clear differences between those trials in whole-body lipid utilisation or blood NEFA concentrations during exercise were shown, suggesting that if protein is consumed in isolation, lipid metabolism may not be supressed *versus* exercise during fasting. Despite this, a time x trial interaction effect was reported for blood glycerol, with lower concentrations in the protein trial, which suggests that ingesting protein before- and during-exercise blunts the net mobilisation of lipids from the adipose tissue compared to fasted exercise. Also, (although the difference was not statistically significant) lipid utilisation rates were ~ 0.10 $g \cdot min^{-1}$ lower with protein ingestion.

In another study, 9 men cycled for 2 h at 70 % $\dot{V}O2$ peak before breakfast or after having consumed whey protein or a leucine-enriched whey gel providing 22 g protein 60 min pre-, 22 g during- and 22 g post-exercise (507). In this study, urinary nitrogen excretion was not measured so a contribution of protein to energy metabolism was not accounted for, so these results should be interpreted with caution. However, no clear differences were shown between trials for whole-body lipid utilisation rates during exercise [(mean ± SEM); whey 0.37 ± 0.26, leucine 0.36 ± 0.24, fasted 0.34 ± 0.24 $g \cdot min^{-1}$] or for blood NEFA concentrations, despite higher blood insulin concentrations in the protein trials. However, as mentioned, a contribution of protein to energy metabolism was not accounted for, and when a similar dose of protein is provided, protein utilisation is increased (508). As the RQ of an average amino acid is 0.807 (67), any increase in protein utilisation with

protein consumption before exercise would decrease the RER and potentially result in an overestimation of lipid utilisation rates, when this is not accounted for. Despite this, a study that did account for a utilisation of protein in estimations of substrate utilisation also reported that consuming protein (20 g) after exercise does not supress rates of whole-body lipid utilisation during subsequent rest or exercise *versus* exercise during morning fasting, whereas whole-body lipid utilisation rates during exercise were decreased when carbohydrate (40 g) was ingested prior [(mean ± SD): fasting: 0.57 ± 0.13; protein 0.52 ± 0.08; carbohydrate 0.44 ± 0.12 $g \cdot min^{-1}$ (509)]. Although confirmatory research is now required, if stimulating a higher rate of lipid utilisation during exercise is important for training-induced changes in oral-glucose insulin sensitivity (**Chapter 6**), eating a protein-rich meal and avoiding dietary carbohydrates before exercise may be an alternative option to exercising during a morning fast. Further investigations of any differences in the stores of lipids utilised during exercise after an overnight fast *versus* after protein ingestion would also be beneficial, in light of the results in **Chapter 6**, showing a blunting of IMTG utilisation with exercise after breakfast.

The extent to which the timing of exercise throughout the day mediates training responses remains to be clarified in humans (and independent of nutrient status). A study in mice used a transcriptomic and metabolic approach to investigate the impact of a single bout of exercise performed in the morning compared to the evening on skeletal muscle metabolism and suggested that global remodelling of skeletal muscle may be altered by the timing of exercise during the day (510). Many biological processes relating to energy metabolism are characterised by daily variations controlled by a central clock located in the hypothalamus and clocks located in peripheral tissues (511). The circadian clock in skeletal muscle is proposed to play an important role in the regulation of muscle metabolism and its regulation may be linked to energy sensors such as AMPK. Peripheral clocks may also be sensitive to feeding (512) and dietary challenges (513). The rodent study suggests that this regulation may extend to exercise, but this now needs to be confirmed in humans. A recent study (514) in humans did suggest that (when all exercise sessions were performed in the fed-state) there may be additional

health benefits to performing exercise in the afternoon *versus* the morning for individuals with T2D. However, in **Chapter 6**, when the timing of exercise *per se* did not differ across exercise groups, nutrient-exercise interactions also mediated adaptations to training. If the timing of exercise during the day also alters these responses (in a fasted- *versus* fed-state), results from studies with exercise in the morning may not be applied to exercise at other times. Together these studies all demonstrate the sensitivity of exercise to the conditions in which it is performed.

Moderate-intensity endurance-type exercise facilitates the biggest increase in lipid utilisation *versus* rest and also the greatest difference in lipid utilisation with exercise and prior breakfast consumption *versus* omission. Indeed, at higher intensities carbohydrates are a greater contributor to metabolism, regardless of whether the exercise is performed in the fed- *versus* fasted-state (249). As such, if a higher rate of lipid utilisation with exercise before nutrient-intake is important for adaptations related to health, this effect may be most apparent with moderate intensity exercise. Nonetheless, future research could investigate the effects of altered nutrient-timing with other forms of exercise that are commonly undertaken in developed countries, as alternatives to endurance-type exercise. Secondly, whether a minimal number of sessions per week need to be performed before *versus* after nutrient-intake for greater increases in oral-glucose insulin sensitivity should be studied (and whether increasing the overall exercise dose performed reduces differences between exercise performed in the fasted *versus* fed-state). Investigating any effects of changing the meal composition or the specific timing of pre-exercise meals before exercise may also provide insights for people who cannot (or prefer not to) exercise during morning fasts. Indeed, whole-body lipid utilisation rates are higher with exercise performed before *versus* after consuming carbohydrates but the magnitude of this difference can also depend on the meal content (e.g. the glycaemic index) and the length of the fast between the last meal consumed and the exercise bout (270). Finally, whether performing exercise before *versus* after breakfast (with no carbohydrate consumed *during* exercise) alters adaptations to training in healthy humans also requires investigation.

The research in this thesis focused on blood glucose regulation but acute bouts of exercise before *versus* after eating may also be more efficacious for lowering lipidemic responses to the meal, due to a greater activation of LPL in blood and skeletal muscle after exercise (515, 516). In **Chapter 6**, glycerol-blanked fasting blood triglyceride concentrations were lowered by performing exercise training but the magnitude of this response did not differ with exercise performed before *versus* after nutrient intake. Other studies that have manipulated nutrient-timing in relation to exercise show either no change in fasting blood lipid concentrations (13, 310) or decreases with training, but with the magnitude of this decrement being similar (11, 12). However, future work could now investigate postprandial lipidaemia with regular exercise performed before *versus* after breakfast.

In this thesis only men were included to remove sex differences as a potential confounding factor for key outcomes that were reported. During endurance-type exercise of the same relative intensity, a contribution of lipids to metabolism is often higher in women, particularly when sex differences in body composition and cardiorespiratory fitness are accounted for (97, 517, 518). A higher rate of lipid utilisation during exercise in women has been attributed to effects of oestrogens, an increased expression of lipid metabolism proteins, a higher intramuscular lipid content and an increased proportion of type I muscle fibres in women *versus* men (519). Pre-menopausal women are also typically more insulin sensitive than men matched for BMI and training status, likely due to differences in oestrogen and adipokine secretion and/or the size of adipose-tissue depots (520). To complicate this further, the contribution of lipids to metabolism and insulin sensitivity in women changes during the menstrual cycle and whilst this can be accounted for during a study, the length of the menstrual cycle is not always uniform. Although confirmatory research is now required, there is no current evidence to suggest that results from this thesis should not be applied to women, as omitting (*versus* consuming) nutrients before exercise increases lipid utilisation in both sexes (67).

Collectively, the research in this thesis has revealed that the timing of meals in relation to exercise potently alters both the short- and longer-term regulation of

glucose metabolism. For meals consumed immediately after cycling exercise consuming a prior breakfast increases plasma glucose appearance rates and glucose disposal rates in healthy men, to the extent that a balance is achieved and plasma glucose concentrations do not clearly differ *versus* when breakfast is omitted. Performing exercise before nutrient intake increases fat utilisation during the activity and this occurs in response to a single bout of exercise and is also sustained throughout a period exercise training. With regular exercise training, consuming nutrients after exercise and performing exercise during a morning fast is more efficacious for improving oral glucose insulin sensitivity and augments intramuscular adaptations relating to energy sensing, glucose transport and the phospholipid composition in overweight men, compared to when carbohydrates are ingested before the exercise. These changes may be due to a higher rate of lipid utilisation during each bout of exercise performed and/or aspects relating to energy sensing in exercised muscle. In healthy men, omitting *versus* consuming breakfast before exercise may be most favourable for creating a negative energy balance over that day. However, after 4-6 weeks of training, any changes in body mass or behavioural components of energy balance do not consistently or clearly differ with exercise before *versus* after (the same) nutrient intake. Therefore, for changes in body mass, the energy deficit from exercise and/or breakfast omission is the main consideration, whilst for the regulation of postprandial glycemia, the specific timing of nutrient intake in relation to exercise has a mediating role, with a heightened response to fasted- exercise. Important next steps include:

1) Characterising substrate use during exercise performed before *versus* after breakfast (with no carbohydrate ingested during exercise) in humans with and without obesity, with a focus on IMTG and muscle glycogen (by using tracer techniques, MRS and/or transmission electron microscopy).
2) Designing exercise interventions for health that include different exercise modes and intensities (e.g. high intensity interval and moderate-intensity endurance-type exercise) and periodising food intake accordingly.
3) Investigating the long-term impact of omitting breakfast and exercising on energy balance and body mass in humans with and without obesity.

REFERENCES FOR NON-PUBLISHED WORK

1. National-Health-Services. Health Survey for England 2017 [internet]. [updated 2018 Dec 4; cited 2019 June 4] Available from: https://digitalnhsuk/data-and-information/publications/statistical/health-survey-for-england/2017.
2. World-Health-Organization. Fact Sheet Number 311: Obesity and Overweight [internet]. [updated 2018 Feb 16; cited 2019 June 4]. Available from: http://www.who.int/mediacentre/factsheets/fs311/en/.
3. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. The Journal of the American Medical Association. 1999;282(16):1523-9.
4. Dobbs R, Sawers C, Thompson F, Manyika J, Woetzel JR, Child P, et al. Overcoming obesity: An initial economic analysis. McKinsey Global Institute, 2014. [internet]. [updated 2014 Nov; cited 2019 June 4]. Available from: https://www.mckinsey.com/mgi/overview
5. Welbourn R, le Roux CW, Owen-Smith A, Wordsworth S, Blazeby JM. Why the NHS should do more bariatric surgery; how much should we do? British Medical Journal. 2016;353:472-7.
6. Reichert FF, Barros AJ, Domingues MR, Hallal PC. The role of perceived personal barriers to engagement in leisure-time physical activity. American Journal of Public Health. 2007;97(3):515-9.
7. Boulé NG, Weisnagel SJ, Lakka TA, Tremblay A, Bergman RN, Rankinen T, et al. Effects of exercise training on glucose homeostasis: the HERITAGE Family Study. Diabetes Care. 2005;28(1):108-14.
8. Wallis GA, Gonzalez JT. Is exercise best served on an empty stomach? Proceedings of the Nutrition Society. 2019;78(1):110-7.
9. Hulver MW, Dohm GL. The molecular mechanism linking muscle fat accumulation to insulin resistance. Proceedings of the Nutrition Society. 2004;63(2):375-80.
10. Enevoldsen L, Simonsen L, Macdonald I, Bülow J. The combined effects of exercise and food intake on adipose tissue and splanchnic metabolism. The Journal of Physiology. 2004;561(3):871-82.
11. Van Proeyen K, Szlufcik K, Nielens H, Pelgrim K, Deldicque L, Hesselink M, et al. Training in the fasted state improves glucose tolerance during fat-rich diet. The Journal of Physiology. 2010;588(21):4289-302.
12. Van Proeyen K, Szlufcik K, Nielens H, Ramaekers M, Hespel P. Beneficial metabolic adaptations due to endurance exercise training in the fasted state. Journal of Applied Physiology. 2011;110(1):236-45.
13. De Bock K, Derave W, Eijnde BO, Hesselink M, Koninckx E, Rose AJ, et al. Effect of training in the fasted state on metabolic responses during exercise with carbohydrate intake. Journal of Applied Physiology. 2008;104(4):1045-55.
14. Chen Y-C, Travers RL, Walhin J-P, Gonzalez JT, Koumanov F, Betts JA, et al. Feeding influences adipose tissue responses to exercise in overweight men. American Journal of Physiology-Endocrinology and Metabolism. 2017;313(1):84-93.
15. Gonzalez JT, Veasey RC, Rumbold PL, Stevenson EJ. Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. British Journal of Nutrition. 2013;110(4):721-32.
16. Bachman JL, Deitrick RW, Hillman AR. Exercising in the Fasted State Reduced 24-Hour Energy Intake in Active Male Adults. Journal of Nutrition and Metabolism. 2016;Epub; DOI: 10.1155/2016/1984198.
17. Hopkins M, Jeukendrup A, King NA, Blundell JE. The relationship between substrate metabolism, exercise, appetite control. Sports Medicine. 2011;41(6):507-21.

18. Hill JO, Wyatt HR, Peters JC. Energy balance and obesity. Circulation. 2012;126(1):126-32.
19. Di Angelantonio E, Bhupathiraju SN, Wormser D, Gao P, Kaptoge S, de Gonzalez AB, et al. Body-mass index and all-cause mortality: individual-participant-data meta-analysis of 239 prospective studies in four continents. The Lancet. 2016;388:776-86.
20. Duncan M, Griffith M, Rutter H, Goldacre MJ. Certification of obesity as a cause of death in England 1979–2006. European Journal of Public Health. 2010;20(6):671-5.
21. World-Health-Organization. Diabetes. [internet]. [updated 2018 Oct 30; cited 2019 June 4]. Available from: http://www.who.int/mediacentre/factsheets/fs312/en/.
22. Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. The Lancet. 2011;378(9793):815-25.
23. Lehnert T, Sonntag D, Konnopka A, Riedel-Heller S, König H-H. Economic costs of overweight and obesity. Best Practice & Research Clinical Endocrinology & Metabolism. 2013;27(2):105-15.
24. Diabetes-UK. Cost of Diabetes [internet]. [updated 2018; cited 2019 June 4]. Available from: http://www.diabetes.co.uk/cost-of-diabetes.html.
25. World-Health-Organization. Global action plan for the prevention and control of noncommunicable diseases 2013-2020. [internet]. [updated 2013; cited 2019 June 4]. Available from: https://www.who.int/nmh/events/ncd_action_plan/en/.
26. Haffner SM. Abdominal adiposity and cardiometabolic risk: do we have all the answers? The American Journal of Medicine. 2007;120(9):10-6.
27. Walhin JP, Richardson JD, Betts JA, Thompson D. Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. The Journal of Physiology. 2013;591(24):6231-43.
28. Bergouignan A, Rudwill F, Simon C, Blanc S. Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies. Journal of Applied Physiology. 2011;111(4):1201-10.
29. Wolfe RR, Chinkes DL. Isotope tracers in metabolic research: principles and practice of kinetic analysis. 2nd ed. Hoboken, New Jersey, US: John Wiley & Sons; 2005.
30. Wasserman DH. Four grams of glucose. American Journal of Physiology-Endocrinology and Metabolism. 2009;296(1):11-21.
31. Frayn KN. Metabolic regulation: a human perspective. 3rd ed. ed. Hoboken, New Jersey, United States: John Wiley & Sons; 2009.
32. Widmaier EP, Raff H, Strang KT, Vander AJ. Vander's human physiology: the mechanisms of body function. 12th ed: Boston: McGraw-Hill Higher Education; 2008.
33. Lavoie C, Ducros F, Bourque J, Langelier H, Chiasson J-L. Glucose metabolism during exercise in man: the role of insulin and glucagon in the regulation of hepatic glucose production and gluconeogenesis. Canadian Journal of Physiology and Pharmacology. 1997;75(1):26-35.
34. Ramnanan C, Edgerton D, Kraft G, Cherrington A. Physiologic action of glucagon on liver glucose metabolism. Diabetes, Obesity and Metabolism. 2011;13:118-25.
35. Prats C, Graham TE, Shearer J. The dynamic life of the glycogen granule. Journal of Biological Chemistry. 2018;293(19):7089-98.
36. Taylor R, Magnusson I, Rothman DL, Cline GW, Caumo A, Cobelli C, et al. Direct assessment of liver glycogen storage by 13C nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. Journal of Clinical Investigation. 1996;97(1):126-32.
37. Banting FG, Best CH. Pancreatic extracts. The Journal of Laboratory and Clinical Medicine. 1922;7(8):464-72.

38. Andrali SS, Sampley ML, Vanderford NL, Özcan S. Glucose regulation of insulin gene expression in pancreatic β-cells. Biochemical Journal. 2008;415(1):1-10.
39. Eliasson L, Abdulkader F, Braun M, Galvanovskis J, Hoppa MB, Rorsman P. Novel aspects of the molecular mechanisms controlling insulin secretion. The Journal of Physiology. 2008;586(14):3313-24.
40. Vilsbøll T, Krarup T, Madsbad S, Holst JJ. Both GLP-1 and GIP are insulinotropic at basal and postprandial glucose levels and contribute nearly equally to the incretin effect of a meal in healthy subjects. Regulatory Peptides. 2003;114:115-21.
41. Muratoglu M, Kuyumjian J, Kalant N. First-pass hepatic uptake and utilization of glucose in the rat. The Biochemical journal. 1986;233(1):245-8.
42. Harada N, Inagaki N. Role of sodium-glucose transporters in glucose uptake of the intestine and kidney. Journal of Diabetes Investigation. 2012;3(4):352-3.
43. DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. Journal of Clinical Investigation. 1985;76(1):149-55.
44. Craig J, Drucker W, Miller M, Woodward Jr H. A prompt effect of exogenous insulin on net hepatic glucose output in man. Metabolism: Clinical and Experimental. 1961;10:212-20.
45. Björntorp P, Sjöström L. Carbohydrate storage in man: speculations and some quantitative considerations. Metabolism. 1978;27(12):1853-65.
46. Ferrannini E, Bjorkman O, Reichard GA, Pilo A, Olsson M, Wahren J, et al. The disposal of an oral glucose load in healthy subjects: a quantitative study. Diabetes. 1985;34(6):580-8.
47. Kelley D, Mitrakou A, Marsh H, Schwenk F, Benn J, Sonnenberg G, et al. Skeletal muscle glycolysis, oxidation, and storage of an oral glucose load. The Journal of Clinical Investigation. 1988;81(5):1563-71.
48. Kandror KV, Pilch PF. Compartmentalization of protein traffic in insulin-sensitive cells. American Journal of Physiology-Endocrinology And Metabolism. 1996;271:1-14.
49. Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nature reviews Molecular cell biology. 2002;3(4):267.
50. Rowland AF, Fazakerley DJ, James DE. Mapping insulin/GLUT4 circuitry. Traffic. 2011;12(6):672-81.
51. Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. American Journal of Physiology-Endocrinology and Metabolism. 2008;295(1):29-37.
52. Treebak JT, Taylor EB, Witczak CA, An D, Toyoda T, Koh H-J, et al. Identification of a novel phosphorylation site on TBC1D4 regulated by AMP-activated protein kinase in skeletal muscle. American Journal of Physiology-Cell Physiology. 2009;298(2):377-85.
53. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, et al. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes. 2006;55(7):2067-76.
54. Hoffman NJ, Elmendorf JS. Signaling, cytoskeletal and membrane mechanisms regulating GLUT4 exocytosis. Trends in Endocrinology and Metabolism. 2011;22(3):110-6.
55. Chiang S-H, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, et al. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature. 2001;410(6831):944-8.
56. Thiebaud D, Jacot E, Defronzo RA, Maeder E, Jequier E, Felber J-P. The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. Diabetes. 1982;31(11):957-63.
57. Srivastava AK, Pandey SK. Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. Molecular & Cellular Biochemistry. 1998;182:135-41.

58. Lafontan M, Langin D. Lipolysis and lipid mobilization in human adipose tissue. Progress in Lipid Research. 2009;48(5):275-97.
59. Bouskila M, Hirshman MF, Jensen J, Goodyear LJ, Sakamoto K. Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 2008;294(1):28-35.
60. Maheux P, Chen Y, Polonsky K, Reaven G. Evidence that insulin can directly inhibit hepatic glucose production. Diabetologia. 1997;40(11):1300-6.
61. Girard J. The inhibitory effects of insulin on hepatic glucose production are both direct and indirect. Diabetes. 2006;55(2):65-9.
62. Raskin P, Fujita Y, Unger RH. Effect of insulin-glucose infusions on plasma glucagon levels in fasting diabetics and nondiabetics. The Journal of Clinical Investigation. 1975;56(5):1132-8.
63. Ishihara H, Maechler P, Gjinovci A, Herrera P-L, Wollheim CB. Islet β-cell secretion determines glucagon release from neighbouring α-cells. Nature Cell Biology. 2003;5(4):330-5.
64. Lewis GF, Vranic M, Giacca A. Glucagon enhances the direct suppressive effect of insulin on hepatic glucose production in humans. American Journal of Physiology-Endocrinology and Metabolism. 1997;272(3):371-8.
65. Pocai A, Obici S, Schwartz GJ, Rossetti L. A brain-liver circuit regulates glucose homeostasis. Cell Metabolism. 2005;1(1):53-61.
66. Sherman WM, Plyley MJ, Sharp RL, Van Handel PJ, McAllister RM, Fink WJ, et al. Muscle glycogen storage and its relationship with water. International Journal of Sports Medicine. 1982;3(1):22-4.
67. Jeukendrup A, Wallis G. Measurement of substrate oxidation during exercise by means of gas exchange measurements. International Journal of Sports Medicine. 2005;26(1):28-37.
68. Nye C, Kim J, Kalhan SC, Hanson RW. Reassessing triglyceride synthesis in adipose tissue. Trends in Endocrinology & Metabolism. 2008;19(10):356-61.
69. Karpe F, Dickmann JR, Frayn KN. Fatty acids, obesity, and insulin resistance: time for a reevaluation. Diabetes. 2011;60(10):2441-9.
70. Vega-López S, Ausman LM, Matthan NR, Lichtenstein AH. Postprandial lipid responses to standard carbohydrates used to determine glycaemic index values. British Journal of Nutrition. 2013;110(10):1782-8.
71. Chavez–Jauregui RN, Mattes RD, Parks EJ. Dynamics of fat absorption and effect of sham feeding on postprandial lipema. Gastroenterology. 2010;139(5):1538-48.
72. Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD. Splanchnic lipolysis in human obesity. The Journal of Clinical Investigation. 2004;113(11):1582-8.
73. Ferrannini E, Camastra S, Coppack S, Fliser D, Golay A, Mitrakou A. Insulin action and non-esterified fatty acids. Proceedings of the Nutrition Society. 1997;56(2):753-61.
74. Jensen MD, Ekberg K, Landau BR. Lipid metabolism during fasting. American Journal of Physiology-Endocrinology and Metabolism. 2001;281(4):789-93.
75. Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E, Sul HS. Regulation of lipolysis in adipocytes. Annual Review of Nutrition. 2007;27:79-101.
76. Brasaemle DL. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. Journal of Lipid Research. 2007;48(12):2547-59.
77. van Hall G, Steensberg A, Sacchetti M, Fischer C, Keller C, Schjerling P, et al. Interleukin-6 stimulates lipolysis and fat oxidation in humans. The Journal of Clinical Endocrinology and Metabolism. 2003;88(7):3005-10.
78. Hardie DG. Regulation of fatty acid synthesis via phosphorylation of acetyl-CoA carboxylase. Progress in Lipid Research. 1989;28(2):117-46.

79. Fery F, d'Attellis N, Balasse E. Mechanisms of starvation diabetes: a study with double tracer and indirect calorimetry. American Journal of Physiology-Endocrinology and Metabolism. 1990;259(6):770-7.
80. Frayn K, Fielding B, Humphreys S, Coppack S. Nutritional influences on human adipose-tissue metabolism. Biochemical Society Transactions. 1996;24(2):422-6.
81. Nurjhan N, Campbell P, Kennedy F, Miles J, Gerich J. Insulin dose-response characteristics for suppression of glycerol release and conversion to glucose in humans. Diabetes. 1986;35(12):1326-31.
82. Wendel AA, Lewin TM, Coleman RA. Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2009;1791(6):501-6.
83. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell. 2006;124(1):35-46.
84. Randle P, Garland P, Hales C, Newsholme E. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. The Lancet. 1963;281(7285):785-9.
85. Boden G, Chen X, Ruiz J, White JV, Rossetti L. Mechanisms of fatty acid-induced inhibition of glucose uptake. The Journal of Clinical Investigation. 1994;93(6):2438-46.
86. Randle PJ. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes and Metabolism Reviews. 1998;14(4):263-83.
87. Goodpaster BH, Sparks LM. Metabolic flexibility in health and disease. Cell Metabolism. 2017;25(5):1027-36.
88. Frayn K. Calculation of substrate oxidation rates in vivo from gaseous exchange. Journal of Applied Physiology. 1983;55(2):628-34.
89. Andres R, Cader G, Zierler KL. The quantitatively minor role of carbohydrate in oxidative metabolism by skeletal muscle in intact man in the basal state. Measurements of oxygen and glucose uptake and carbon dioxide and lactate production in the forearm. The Journal of Clinical Investigation. 1956;35(6):671-82.
90. Kelley DE, Goodpaster B, Wing RR, Simoneau J-A. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. American Journal of Physiology-Endocrinology and Metabolism. 1999;277(6):1130-41.
91. Kelley DE, Simoneau J-A. Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. Journal of Clinical Investigation. 1994;94(6):2349–56.
92. Perseghin G, Bonfanti R, Magni S, Lattuada G, De Cobelli F, Canu T, et al. Insulin resistance and whole body energy homeostasis in obese adolescents with fatty liver disease. American Journal of Physiology-Endocrinology and Metabolism. 2006;291(4):697-703.
93. Simoneau J-a, Veerkamp JH, Turcotte LP, Kelley DE. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. The FASEB Journal. 1999;13(14):2051-60.
94. Ara I, Larsen S, Stallknecht B, Guerra B, Morales-Alamo D, Andersen J, et al. Normal mitochondrial function and increased fat oxidation capacity in leg and arm muscles in obese humans. International Journal of Obesity. 2011;35(1):99.
95. Andersen P, Saltin B. Maximal perfusion of skeletal muscle in man. The Journal of Physiology. 1985;366(1):233-49.
96. Segal KR, Gutin B, Nyman AM, Pi-Sunyer FX. Thermic effect of food at rest, during exercise, and after exercise in lean and obese men of similar body weight. The Journal of Clinical Investigation. 1985;76(3):1107-12.

97. Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. Journal of Applied Physiology. 2005;98(1):160-7.
98. Falta W, Boller R. Insulärer und insulinresistenter Diabetes. Klinische Wochenschrift. 1931;10(10):438-43.
99. Reaven GM. Role of insulin resistance in human disease. Diabetes. 1988;37(12):1595-607.
100. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of insulin resistance in skeletal muscle. BioMed Research International. 2010;Epub: doi: 10.1155/2010/476279.
101. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006;444(7121):840-6.
102. Bogardus C, Lillioja S, Mott D, Reaven G, Kashiwagi A, Foley J. Relationship between obesity and maximal insulin-stimulated glucose uptake in vivo and in vitro in Pima Indians. The Journal of Clinical Investigation. 1984;73(3):800-5.
103. Eriksson J, Koranyi L, Bourey R, Schalin-Jäntti C, Widen E, Mueckler M, et al. Insulin resistance in type 2 (non-insulin-dependent) diabetic patients and their relatives is not associated with a defect in the expression of the insulin-responsive glucose transporter (GLUT-4) gene in human skeletal muscle. Diabetologia. 1992;35(2):143-7.
104. Bonadonna RC, Del Prato S, Saccomani MP, Bonora E, Gulli G, Ferrannini E, et al. Transmembrane glucose transport in skeletal muscle of patients with non-insulin-dependent diabetes. The Journal of Clinical Investigation. 1993;92(1):486-94.
105. Damsbo P, Vaag A, Hother-Nielsen O, Beck-Nielsen H. Reduced glycogen synthase activity in skeletal muscle from obese patients with and without type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia. 1991;34(4):239-45.
106. Caro JF, Sinha MK, Raju S, Ittoop O, Pories WJ, Flickinger EG, et al. Insulin receptor kinase in human skeletal muscle from obese subjects with/without noninsulin dependent diabetes. The Journal of Clinical Investigation. 1987;79(5):1330-7.
107. Olefsky JM, Reaven GM. Insulin binding in diabetes: relationships with plasma insulin levels and insulin sensitivity. Diabetes. 1977;26(7):680-8.
108. Klein HH, Vestergaard H, Kotzke G, Pedersen O. Elevation of serum insulin concentration during euglycemic hyperinsulinemic clamp studies leads to similar activation of insulin receptor kinase in skeletal muscle of subjects with and without NIDDM. Diabetes. 1995;44(11):1310-7.
109. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, et al. Insulin resistance differentially affects the PI 3-kinase–and MAP kinase–mediated signaling in human muscle. The Journal of Clinical Investigation. 2000;105(3):311-20.
110. Brown MS, Goldstein JL. Selective versus total insulin resistance: a pathogenic paradox. Cell Metabolism. 2008;7(2):95-6.
111. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. The Journal of Clinical Investigation. 2016;126(1):12-22.
112. Qu X, Seale J, Donnelly R. Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats-effects of feeding. Journal of Endocrinology. 1999;162(2):207-14.
113. Magkos F, Su X, Bradley D, Fabbrini E, Conte C, Eagon JC, et al. Intrahepatic diacylglycerol content is associated with hepatic insulin resistance in obese subjects. Gastroenterology. 2012;142(7):1444-6.
114. Jensen MD, Caruso M, Heiling V, Miles JM. Insulin regulation of lipolysis in nondiabetic and IDDM subjects. Diabetes. 1989;38(12):1595-601.
115. Turner N, Kowalski G, Leslie S, Risis S, Yang C, Lee-Young R, et al. Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. Diabetologia. 2013;56(7):1638-48.

116. Chen D, Liess C, Poljak A, Xu A, Zhang J, Thoma C, et al. Phenotypic characterization of insulin-resistant and insulin-sensitive obesity. The Journal of Clinical Endocrinology and Metabolism. 2015;100(11):4082-91.
117. McLaughlin T, Lamendola C, Liu A, Abbasi F. Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. The Journal of Clinical Endocrinology and Metabolism. 2011;96(11):1756-60.
118. Kovalik J-P, Slentz D, Stevens RD, Kraus WE, Houmard JA, Nicoll JB, et al. Metabolic remodeling of human skeletal myocytes by cocultured adipocytes depends on the lipolytic state of the system. Diabetes. 2011;60(7):1882-93.
119. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.
120. Pan D, Lillioja S, Kriketos A, Milner M, Baur L, Bogardus C, et al. Skeletal muscle triglyceride levels are inversely related to insulin action. Diabetes. 1997;46(6):983-8.
121. Phillips D, Caddy S, Ilic V, Fielding B, Frayn K, Borthwick A, et al. Intramuscular triglyceride and muscle insulin sensitivity: evidence for a relationship in nondiabetic subjects. Metabolism. 1996;45(8):947-50.
122. Kelley DE, Slasky BS, Janosky J. Skeletal muscle density: effects of obesity and non-insulin-dependent diabetes mellitus. The American Journal of Clinical Nutrition. 1991;54(3):509-15.
123. Boesch C, Slotboom J, Hoppeler H, Kreis R. In vivo determination of intra-myocellular lipids in human muscle by means of localized 1H-MR-spectroscopy. Magnetic Resonance in Medicine. 1997;37(4):484-93.
124. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, et al. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. Diabetes. 1999;48(8):1600-6.
125. Krssak M, Petersen KF, Dresner A, DiPietro L, Vogel S, Rothman D, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. Diabetologia. 1999;42(1):113-6.
126. Savage DB, Watson L, Carr K, Adams C, Brage S, Chatterjee KK, et al. Accumulation of saturated intramyocellular lipid is associated with insulin resistance. Journal of Lipid Research. 2019:Epub doi: 10.1194/jlr.M091942.
127. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. The Journal of Clinical Endocrinology and Metabolism. 2001;86(12):5755-61.
128. Moro C, Bajpeyi S, Smith SR. Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity. American Journal of Physiology-Endocrinology and Metabolism. 2008;294(2):203-13.
129. Summers SA. Ceramides in insulin resistance and lipotoxicity. Progress in Lipid Research. 2006;45(1):42-72.
130. Timmers S, Schrauwen P, de Vogel J. Muscular diacylglycerol metabolism and insulin resistance. Physiology & Behavior. 2008;94(2):242-51.
131. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. The Lancet. 2010;375(9733):2267-77.
132. Schmitz-Peiffer C, Craig DL, Biden TJ. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. Journal of Biological Chemistry. 1999;274(34):24202-10.
133. Bosma M, Kersten S, Hesselink MK, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. Progress in Lipid Research. 2012;51(1):36-49.

134. Harayama T, Riezman H. Understanding the diversity of membrane lipid composition. Nature Reviews Molecular Cell Biology. 2018;19(5):281-96.
135. Wigger L, Cruciani-Guglielmacci C, Nicolas A, Denom J, Fernandez N, Fumeron F, et al. Plasma dihydroceramides are diabetes susceptibility biomarker candidates in mice and humans. Cell Reports. 2017;18(9):2269-79.
136. Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW. Influence of dietary fat composition on development of insulin resistance in rats: relationship to muscle triglyceride and ω-3 fatty acids in muscle phospholipid. Diabetes. 1991;40(2):280-9.
137. Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. New England Journal of Medicine. 1993;328(4):238-44.
138. Field CJ, Ryan E, Thomson A, Clandinin M. Dietary fat and the diabetic state alter insulin binding and the fatty acyl composition of the adipocyte plasma membrane. Biochemical Journal. 1988;253(2):417-24.
139. Yorek M, Leeney E, Dunlap J, Ginsberg B. Effect of fatty acid composition on insulin and IGF-I binding in retinoblastoma cells. Investigative Ophthalmology and Visual Science. 1989;30(10):2087-92.
140. Ginsberg BH, Jabour J, Spector AA. Effect of alterations in membrane lipid unsaturation on the properties of the insulin receptor of Ehrlich ascites cells. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1982;690(2):157-64.
141. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proceedings of the National Academy of Sciences. 1997;94(9):4318-23.
142. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of Clinical Investigation. 2003;112(12):1821-30.
143. Peraldi P, Spiegelman B. TNF-α and insulin resistance: summary and future prospects. Molecular and Cellular Biochemistry. 1998;182(1-2):169-75.
144. Hotamisligil G. The role of TNFα and TNF receptors in obesity and insulin resistance. Journal of Internal Medicine. 1999;245(6):621-5.
145. Hellmann J, Tang Y, Kosuri M, Bhatnagar A, Spite M. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. The FASEB Journal. 2011;25(7):2399-407.
146. Goldfine AB, Fonseca V, Jablonski KA, Pyle L, Staten MA, Shoelson SE. The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. Annals of Internal Medicine. 2010;152(6):346-57.
147. Goossens GH, Bizzarri A, Venteclef N, Essers Y, Cleutjens JP, Konings E, et al. Increased Adipose Tissue Oxygen Tension in Obese Compared With Lean Men Is Accompanied by Insulin Resistance, Impaired Adipose Tissue Capillarization, and InflammationClinical Perspective. Circulation. 2011;124(1):67-76.
148. Nishimura S, Manabe I, Nagai R. Adipose tissue inflammation in obesity and metabolic syndrome. Discovery medicine. 2009;8(41):55-60.
149. Tricò D, Natali A, Arslanian S, Mari A, Ferrannini E. Identification, pathophysiology, and clinical implications of primary insulin hypersecretion in nondiabetic adults and adolescents. JCI Insight. 2018;3(24):Epub: doi: 10.1172/jci.insight.124912.
150. Rizza R, Mandarino L, Genest J, Baker B, Gerich J. Production of insulin resistance by hyperinsulinaemia in man. Diabetologia. 1985;28(2):70-5.
151. Hall KD. Modeling Metabolic Adaptations and Energy Regulation in Humans. Annual Review of Nutrition. 2012;32(1):35-54.

152. Odeleye OE, de Courten M, Pettitt DJ, Ravussin E. Fasting hyperinsulinemia is a predictor of increased body weight gain and obesity in Pima Indian children. Diabetes. 1997;46(8):1341-5.
153. Blüher M, Michael MD, Peroni OD, Ueki K, Carter N, Kahn BB, et al. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Developmental Cell. 2002;3(1):25-38.
154. Templeman NM, Clee SM, Johnson JD. Suppression of hyperinsulinaemia in growing female mice provides long-term protection against obesity. Diabetologia. 2015;58(10):2392-402.
155. Mehran AE, Templeman NM, Brigidi GS, Lim GE, Chu K-Y, Hu X, et al. Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metabolism. 2012;16(6):723-37.
156. Ferrannini E, Barrett E, Bevilacqua S, DeFronzo RA. Effect of fatty acids on glucose production and utilization in man. Journal of Clinical Investigation. 1983;72(5):1737-47.
157. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes. 2005;54(1):8-14.
158. Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, et al. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. Diabetes. 2008;57(11):2943-9.
159. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002;51(10):2944-50.
160. Sparks LM, Xie H, Koza RA, Mynatt R, Hulver MW, Bray GA, et al. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes. 2005;54(7):1926-33.
161. Holloszy JO. “Deficiency” of mitochondria in muscle does not cause insulin resistance. Diabetes. 2013;62(4):1036-40.
162. Hancock CR, Han D-H, Chen M, Terada S, Yasuda T, Wright DC, et al. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. Proceedings of the National Academy of Sciences. 2008;105(22):7815-20.
163. Garcia-Roves P, Huss JM, Han D-H, Hancock CR, Iglesias-Gutierrez E, Chen M, et al. Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. Proceedings of the National Academy of Sciences. 2007;104(25):10709-13.
164. Zechner C, Lai L, Zechner JF, Geng T, Yan Z, Rumsey JW, et al. Total skeletal muscle PGC-1 deficiency uncouples mitochondrial derangements from fiber type determination and insulin sensitivity. Cell Metabolism. 2010;12(6):633-42.
165. Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, et al. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca2+. American Journal of Physiology-Endocrinology and Metabolism. 2002;282(5):1008-13.
166. Martínez-González MÁ, Martinez JA, Hu F, Gibney M, Kearney J. Physical inactivity, sedentary lifestyle and obesity in the European Union. International Journal of Obesity. 1999;23(11):1192.
167. Arner P, Bernard S, Salehpour M, Possnert G, Liebl J, Steier P, et al. Dynamics of human adipose lipid turnover in health and metabolic disease. Nature. 2011;478(7367):110-3.
168. Frayn K, Bernard S, Spalding K, Arner P. Adipocyte Triglyceride Turnover Is Independently Associated With Atherogenic Dyslipidemia. Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease. 2012;1(6):e003467.

169. Shaw CS, Clark J, Wagenmakers AJM. The Effect of Exercise and Nutrition on Intramuscular Fat Metabolism and Insulin Sensitivity. Annual Review of Nutrition. 2010;30(1):13-34.
170. Moller DE, Yokota A, Flier JS. Normal insulin-receptor cDNA sequence in Pima Indians with NIDDM. Diabetes. 1989;38(11):1496-500.
171. Kusari J, Verma U, Buse J, Henry R, Olefsky J. Analysis of the gene sequences of the insulin receptor and the insulin-sensitive glucose transporter (GLUT-4) in patients with common-type non-insulin-dependent diabetes mellitus. The Journal of Clinical Investigation. 1991;88(4):1323-30.
172. Choi W-H, O'Rahilly S, Buse JB, Rees A, Morgan R, Flier JS, et al. Molecular scanning of insulin-responsive glucose transporter (GLUT4) gene in NIDDM subjects. Diabetes. 1991;40(12):1712-8.
173. Echwald SM, Bjørbaek C, Hansen T, Clausen JO, Vestergaard H, Zierath JR, et al. Identification of four amino acid substitutions in hexokinase II and studies of relationships to NIDDM, glucose effectiveness, and insulin sensitivity. Diabetes. 1995;44(3):347-53.
174. Laakso M, Malkki M, Kekäläinen P, Kuusisto J, Deeb S. Polymorphisms of the human hexokinase II gene: lack of association with NIDDM and insulin resistance. Diabetologia. 1995;38(5):617-22.
175. Lehto M, Huang X, Davis E, Le Beau M, Laurila E, Eriksson K, et al. Human hexokinase II gene: exon-intron organization, mutation screening in NIDDM, and its relationship to muscle hexokinase activity. Diabetologia. 1995;38(12):1466-74.
176. Majer M, Mott D, Mochizuki H, Rowles J, Pedersen O, Knowler W, et al. Association of the glycogen synthase locus on 19q13 with NIDDM in Pima Indians. Diabetologia. 1996;39(3):314-21.
177. Blackburn G. Effect of degree of weight loss on health benefits. Obesity Research. 1995;3(2):Epub: https://doi.org/10.1002/j.550-8528.1995.tb00466.x.
178. Most J, Tosti V, Redman LM, Fontana L. Calorie restriction in humans: an update. Ageing Research Reviews. 2017;39:36-45.
179. Murphy JC, McDaniel JL, Mora K, Villareal DT, Fontana L, Weiss EP. Preferential reductions in intermuscular and visceral adipose tissue with exercise-induced weight loss compared with calorie restriction. Journal of Applied Physiology. 2011;112(1):79-85.
180. Toledo FG, Menshikova EV, Azuma K, Radiková Z, Kelley CA, Ritov VB, et al. Mitochondrial capacity in skeletal muscle is not stimulated by weight loss, despite increases in insulin action and decreases in intramyocellular lipid content. Diabetes. 2008;57(4):987-94.
181. Burke LM, Hawley JA. Effects of short-term fat adaptation on metabolism and performance of prolonged exercise. Medicine and Science in Sports and Exercise. 2002;34(9):1492-8.
182. Bachmann OP, Dahl DB, Brechtel K, Machann J, Haap M, Maier T, et al. Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. Diabetes. 2001;50(11):2579-84.
183. Bonen A, Holloway GP, Tandon NN, Han X-X, McFarlan J, Glatz JF, et al. Cardiac and skeletal muscle fatty acid transport and transporters and triacylglycerol and fatty acid oxidation in lean and Zucker diabetic fatty rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2009;297(4):1202-12.
184. Holloway GP, Benton CR, Mullen KL, Yoshida Y, Snook LA, Han X-X, et al. In obese rat muscle transport of palmitate is increased and is channeled to triacylglycerol storage despite an increase in mitochondrial palmitate oxidation. American Journal of Physiology-Endocrinology and Metabolism. 2009;296(4):738-47.
185. Hall KD, Chung ST. Low-carbohydrate diets for the treatment of obesity and type 2 diabetes. Current Opinion Clinical Nutrition & Metabolic Care. 2018;21:308-12.

186. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. The American Journal of Clinical Nutrition. 2005;81(2):341-54.
187. MacLean PS, Bergouignan A, Cornier M-A, Jackman MR. Biology's response to dieting: the impetus for weight regain. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2011;301(3):581-600.
188. Franz MJ, VanWormer JJ, Crain AL, Boucher JL, Histon T, Caplan W, et al. Weight-loss outcomes: a systematic review and meta-analysis of weight-loss clinical trials with a minimum 1-year follow-up. Journal of the American Dietetic Association. 2007;107(10):1755-67.
189. Wing RR, Hill JO. Successful weight loss maintenance. Annual Review of Nutrition. 2001;21(1):323-41.
190. Ewbank PP, Darga LL, Lucas CP. Physical activity as a predictor of weight maintenance in previously obese subjects. Obesity Research. 1995;3(3):257-63.
191. Steig AJ, Jackman MR, Giles ED, Higgins JA, Johnson GC, Mahan C, et al. Exercise reduces appetite and traffics excess nutrients away from energetically efficient pathways of lipid deposition during the early stages of weight regain. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2011;301(3):656-67.
192. Tremblay MS, Colley RC, Saunders TJ, Healy GN, Owen N. Physiological and health implications of a sedentary lifestyle. Applied Physiology, Nutrition and Metabolism. 2010;35(6):725-40.
193. Colberg SR, Sigal RJ, Fernhall B, Regensteiner JG, Blissmer BJ, Rubin RR, et al. Exercise and type 2 diabetes: the American College of Sports Medicine and the American Diabetes Association: joint position statement. Diabetes Care. 2010;33(12):147-67.
194. Richter EA, Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. Physiological Reviews. 2013;93(3):993-1017.
195. Fatone C, Guescini M, Balducci S, Battistoni S, Settequattrini A, Pippi R, et al. Two weekly sessions of combined aerobic and resistance exercise are sufficient to provide beneficial effects in subjects with Type 2 diabetes mellitus and metabolic syndrome. Journal of Endocrinological Investigation. 2010;33(7):489-95.
196. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. The New England Journal of Medicine. 2002;346(6):393-403.
197. Snowling NJ, Hopkins WG. Effects of different modes of exercise training on glucose control and risk factors for complications in type 2 diabetic patients: a meta-analysis. Diabetes Care. 2006;29(11):2518-27.
198. Sylow L, Richter EA. Current advances in our understanding of exercise as medicine in metabolic disease. Current Opinion in Physiology. 2019;Epub: https://doi.org/10.1016/j.cophys.2019.04.008.
199. Jeukendrup AE, Raben A, Gijsen A, Stegen JH, Brouns F, Saris WH, et al. Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion. The Journal of Physiology. 1999;515(2):579-89.
200. Hawley J, Lessard S. Exercise training-induced improvements in insulin action. Acta Physiologica. 2008;192(1):127-35.
201. Martin I, Katz A, Wahren J. Splanchnic and muscle metabolism during exercise in NIDDM patients. American Journal of Physiology-Endocrinology and Metabolism. 1995;269(3):E583-E90.
202. Cokorinos EC, Delmore J, Reyes AR, Albuquerque B, Kjøbsted R, Jørgensen NO, et al. Activation of skeletal muscle AMPK promotes glucose disposal and glucose lowering in non-human primates and mice. Cell Metabolism. 2017;25(5):1147-59.

203. Maehlum S, Felig P, Wahren J. Splanchnic glucose and muscle glycogen metabolism after glucose feeding during postexercise recovery. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1978;235(3):255-60.
204. Kochan RG, Lamb DR, Lutz SA, Perrill CV, Reimann EM, Schlender KK. Glycogen synthase activation in human skeletal muscle: effects of diet and exercise. American Journal of Physiology-Endocrinology And Metabolism. 1979;236(6):660-7.
205. Ivy J, Kuo CH. Regulation of GLUT4 protein and glycogen synthase during muscle glycogen synthesis after exercise. Acta Physiologica Scandinavica. 1998;162(3):295-304.
206. Goodyear LJ, Hirshman MF, King PA, Horton ED, Thompson CM, Horton ES. Skeletal muscle plasma membrane glucose transport and glucose transporters after exercise. Journal of Applied Physiology. 1990;68(1):193-8.
207. Richter EA, Mikines K, Galbo H, Kiens B. Effect of exercise on insulin action in human skeletal muscle. Journal of Applied Physiology. 1989;66(2):876-85.
208. Devlin J, Horton E. Effects of prior high-intensity exercise on glucose metabolism in normal and insulin-resistant men. Diabetes. 1985;34(10):973-9.
209. Hansen PA, Nolte LA, Chen MM, Holloszy JO. Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. Journal of Applied Physiology. 1998;85(4):1218-22.
210. Geiger PC, Han DH, Wright DC, Holloszy JO. How muscle insulin sensitivity is regulated: testing of a hypothesis. American Journal of Physiology-Endocrinology and Metabolism. 2006;291(6):1258-63.
211. Ploug T, Van Deurs B, Ai H, Cushman SW, Ralston E. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. The Journal of Cell Biology. 1998;142(6):1429-46.
212. Lohmann D, Liebold F, Heilmann W, Senger H, Pohl A. Diminished insulin response in highly trained athletes. Metabolism. 1978;27(5):521-4.
213. Björntorp P, Fahlén M, Grimby G, Gustafson A, Holm J, Renström P, et al. Carbohydrate and lipid metabolism in middle-aged, physically well-trained men. Metabolism. 1972;21(11):1037-44.
214. Tonino RP. Effect of physical training on the insulin resistance of aging. American Journal of Physiology-Endocrinology And Metabolism. 1989;256(3):E352-E6.
215. Borghouts L, Keizer H. Exercise and insulin sensitivity: a review. International Journal of Sports Medicine. 2000;21(1):1-12.
216. Houmard JA, Shaw CD, Hickey MS, Tanner CJ. Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in human skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 1999;277(6):1055-60.
217. Kirwan JP, del Aguila LF, Hernandez JM, Williamson DL, O'Gorman DJ, Lewis R, et al. Regular exercise enhances insulin activation of IRS-1-associated PI3-kinase in human skeletal muscle. Journal of Applied Physiology. 2000;88(2):797-803.
218. Frøsig C, Jørgensen SB, Hardie DG, Richter EA, Wojtaszewski JF. 5′-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 2004;286(3):411-7.
219. Goodyear P, Laurie J, Kahn M, Barbara B. Exercise, glucose transport, and insulin sensitivity. Annual Review of Medicine. 1998;49(1):235-61.
220. Leturque A, Loizeau M, Vaulont S, Salminen M, Girard J. Improvement of Insulin Action in Diabetic Transgenic Mice Selectively Overexpressing GLUT4 in Skeletal Muscle. Diabetes. 1996;45(1):23-7.
221. Yan Z, Okutsu M, Akhtar YN, Lira VA. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. Journal of Applied Physiology. 2011;110(1):264-74.

222. Laakso M, Edelman SV, Brechtel G, Baron AD. Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. Diabetes. 1992;41(9):1076-83.
223. Miller W, Sherman W, Ivy J. Effect of strength training on glucose tolerance and post-glucose insulin response. Medicine and Science in Sports and Exercise. 1984;16(6):539-43.
224. Oshida Y, Yamanouchi K, Hayamizu S, Sato Y. Long-term mild jogging increases insulin action despite no influence on body mass index or VO2 max. Journal of Applied Physiology. 1989;66(5):2206-10.
225. Coyle EF. Substrate utilization during exercise in active people. The American Journal of Clinical Nutrition. 1995;61(4):968-79.
226. van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, Wagenmakers AJ. The effects of increasing exercise intensity on muscle fuel utilisation in humans. The Journal of Physiology. 2001;536(1):295-304.
227. Goodpaster BH, Wolfe RR, Kelley DE. Effects of obesity on substrate utilization during exercise. Obesity Research. 2002;10(7):575-84.
228. Horowitz JF, Klein S. Oxidation of nonplasma fatty acids during exercise is increased in women with abdominal obesity. Journal of Applied Physiology. 2000;89(6):2276-82.
229. Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, Hamadeh MJ. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2007;292(3):1271-8.
230. Little JP, Gillen JB, Percival ME, Safdar A, Tarnopolsky MA, Punthakee Z, et al. Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. Journal of Applied Physiology. 2011;111(6):1554-60.
231. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 1999;98(1):115-24.
232. Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, et al. The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle. Proceedings of the National Academy of Sciences. 2009;106(50):21401-6.
233. Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA, Kiens B. Isoform-specific and exercise intensity-dependent activation of 5′-AMP-activated protein kinase in human skeletal muscle. The Journal of Physiology. 2000;528(1):221-6.
234. Amati F, Dubé JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, et al. Skeletal Muscle Triglycerides, Diacylglycerols, and Ceramides in Insulin Resistance Another Paradox in Endurance-Trained Athletes? Diabetes. 2011;60(10):2588-97.
235. Irrcher I, Adhihetty PJ, Joseph A-M, Ljubicic V, Hood DA. Regulation of mitochondrial biogenesis in muscle by endurance exercise. Sports Medicine. 2003;33(11):783-93.
236. Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. Journal of Applied Physiology. 1984;56(4):831-8.
237. Gollnick PD, Saltin B. Significance of skeletal muscle oxidative enzyme enhancement with endurance training. Clinical Physiology. 1982;2(1):1-12.
238. Hoppeler H, Howald H, Conley K, Lindstedt SL, Claassen H, Vock P, et al. Endurance training in humans: aerobic capacity and structure of skeletal muscle. Journal of Applied Physiology. 1985;59(2):320-7.
239. Goodpaster BH, Katsiaras A, Kelley DE. Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity. Diabetes. 2003;52(9):2191-7.

240. Daemen S, Gemmink A, Brouwers B, Meex RC, Huntjens PR, Schaart G, et al. Distinct lipid droplet characteristics and distribution unmask the apparent contradiction of the athlete's paradox. Molecular Metabolism. 2018;17:71-81.
241. Nielsen J, Mogensen M, Vind BF, Sahlin K, Højlund K, Schrøder HD, et al. Increased subsarcolemmal lipids in type 2 diabetes: effect of training on localization of lipids, mitochondria, and glycogen in sedentary human skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 2009;298(3):E706-E13.
242. Kesaniemi YA, Danforth E, Jensen MD, Kopelman PG, LefÈbvre P, Reeder BA. Dose-response issues concerning physical activity and health: an evidence-based symposium. Medicine and Science in Sports and Exercise. 2001;33(6):351-8.
243. Korkiakangas EE, Alahuhta MA, Laitinen JH. Barriers to regular exercise among adults at high risk or diagnosed with type 2 diabetes: a systematic review. Health Promotion International. 2009;24(4):416-27.
244. de Lannoy L, Clarke J, Stotz PJ, Ross R. Effects of intensity and amount of exercise on measures of insulin and glucose: Analysis of inter-individual variability. PloS one. 2017;12(5):e0177095.
245. Atkinson G, Batterham AM. True and false interindividual differences in the physiological response to an intervention. Experimental Physiology. 2015;100:577-88.
246. Lemon P, Nagle F. Effects of exercise on protein and amino acid metabolism. Medicine and Science in Sports and Exercise. 1980;13(3):141-9.
247. McMurray R, Forsythe W, Mar M, Hardy CJ. Exercise intensity-related responses of beta-endorphin and catecholamines. Medicine and Science in Sports and Exercise. 1987;19(6):570-4.
248. Steensberg A, Van Hall G, Osada T, Sacchetti M, Saltin B, Pedersen BK. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. The Journal of Physiology. 2000;529(1):237-42.
249. Bergman BC, Brooks GA. Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. Journal of Applied Physiology. 1999;86(2):479-87.
250. Romijn J, Coyle E, Sidossis L, Gastaldelli A, Horowitz J, Endert E, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. American Journal of Physiology-Endocrinology and Metabolism. 1993;265(3):380-91.
251. Hall KD, Guo J. Obesity Energetics: Body Weight Regulation and the Effects of Diet Composition. Gastroenterology. 2017;152(7):1718-27.
252. Walker M, Cooper B, Elliott C, Reed J, Orskov H, Alberti K. Role of plasma non-esterified fatty acids during and after exercise. Clinical Science. 1991;81(3):319-25.
253. Romijn J, Coyle E, Sidossis L, Zhang X, Wolfe R. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. Journal of Applied Physiology. 1995;79(6):1939-45.
254. De Glisezinski I, Larrouy D, Bajzova M, Koppo K, Polak J, Berlan M, et al. Adrenaline but not noradrenaline is a determinant of exercise-induced lipid mobilization in human subcutaneous adipose tissue. The Journal of Physiology. 2009;587(13):3393-404.
255. Dempsey JA, Harms CA, Ainsworth DM. Respiratory muscle perfusion and energetics during exercise. Medicine and Science in Sports and Exercise. 1996;28(9):1123-8.
256. Kiens B, Roemen TH, van der Vusse GJ. Muscular long-chain fatty acid content during graded exercise in humans. American Journal of Physiology-Endocrinology and Metabolism. 1999;276(2):352-7.
257. Gastin PB. Energy system interaction and relative contribution during maximal exercise. Sports Medicine. 2001;31(10):725-41.

258. Coyle E, Jeukendrup A, Wagenmakers A, Saris W. Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. American Journal of Physiology-Endocrinology And Metabolism. 1997;273(2):268-75.
259. Helge JW, Fraser A, Kriketos AD, Jenkins A, Calvert G, Ayre KJ, et al. Interrelationships between muscle fibre type, substrate oxidation and body fat. International Journal of Obesity. 1999;23(9):986-91.
260. Goedecke JH, Gibson ASC, Grobler L, Collins M, Noakes TD, Lambert EV. Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes. American Journal of Physiology-Endocrinology and Metabolism. 2000;279(6):1325-34.
261. Bureau-of-Labor-Statistics. Sports and Exercise [internet]. [updated 2008 May; cited 2019 June 4] Available from: http://www.bls.gov/spotlight/2008/sports/.
262. Krogh A, Lindhard J. The relative value of fat and carbohydrate as sources of muscular energy: with appendices on the correlation between standard metabolism and the respiratory quotient during rest and work. Biochemical Journal. 1920;14(3):290-363.
263. Montain SJ, Hopper M, Coggan AR, Coyle EF. Exercise metabolism at different time intervals after a meal. Journal of Applied Physiology. 1991;70(2):882-8.
264. Vieira AF, Costa RR, Macedo RCO, Coconcelli L, Kruel LFM. Effects of aerobic exercise performed in fasted v. fed state on fat and carbohydrate metabolism in adults: a systematic review and meta-analysis. British Journal of Nutrition. 2016;116:1153-64.
265. De Bock K, Richter EA, Russell A, Eijnde BO, Derave W, Ramaekers M, et al. Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. The Journal of Physiology. 2005;564(2):649-60.
266. Horowitz JF, Mora-Rodriguez R, Byerley LO, Coyle EF. Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. American Journal of Physiology-Endocrinology and Metabolism. 1997;273(4):768-75.
267. Izumida Y, Yahagi N, Takeuchi Y, Nishi M, Shikama A, Takarada A, et al. Glycogen shortage during fasting triggers liver–brain–adipose neurocircuitry to facilitate fat utilization. Nature Communications. 2013;4:Epub: doi 10.1038/ncomms3316.
268. Bergström J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. Acta Physiologica Scandinavica. 1967;71(2):140-50.
269. Williams C, Brewer J, Walker M. The effect of a high carbohydrate diet on running performance during a 30-km treadmill time trial. European Journal of Applied Physiology and Occupational Physiology. 1992;65(1):18-24.
270. Haxhi J, Scotto di Palumbo A, Sacchetti M. Exercising for metabolic control: is timing important? Annals of Nutrition and Metabolism. 2012;62(1):14-25.
271. Brooks GA, Donovan CM. Effect of endurance training on glucose kinetics during exercise. American Journal of Physiology-Endocrinology And Metabolism. 1983;244(5):505-12.
272. Febbraio MA, Keenan J, Angus DJ, Campbell SE, Garnham AP. Preexercise carbohydrate ingestion, glucose kinetics, and muscle glycogen use: effect of the glycemic index. Journal of Applied Physiology. 2000;89(5):1845-51.
273. Costill D, Coyle E, Dalsky G, Evans W, Fink W, Hoopes D. Effects of elevated plasma FFA and insulin on muscle glycogen usage during exercise. Journal of Applied Physiology. 1977;43(4):695-9.
274. Poirier P, Tremblay A, Catellier C, Tancrède G, Garneau C, Nadeau A. Impact of time interval from the last meal on glucose response to exercise in subjects with type 2 diabetes. The Journal of Clinical Endocrinology & Metabolism. 2000;85(8):2860-4.
275. Poirier P, Mawhinney S, Grondin L, Tremblay A, Broderick T, ClÉroux J, et al. Prior meal enhances the plasma glucose lowering effect of exercise in type 2 diabetes. Medicine and Science in Sports and Exercise. 2001;33(8):1259-64.

276. Abdul-Ghani MA, Lyssenko V, Tuomi T, DeFronzo RA, Groop L. The shape of plasma glucose concentration curve during OGTT predicts future risk of type 2 diabetes. Diabetes and Metabolism Research and Reviews. 2010;26(4):280-6.
277. Cavalot F, Petrelli A, Traversa M, Bonomo K, Fiora E, Conti M, et al. Postprandial blood glucose is a stronger predictor of cardiovascular events than fasting blood glucose in type 2 diabetes mellitus, particularly in women: lessons from the San Luigi Gonzaga Diabetes Study. The Journal of Clinical Endocrinology and Metabolism. 2006;91(3):813-9.
278. Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. Diabetes. 1999;48(5):937-42.
279. Rose AJ, Howlett K, King DS, Hargreaves M. Effect of prior exercise on glucose metabolism in trained men. American Journal of Physiology-Endocrinology and Metabolism. 2001;281(4):766-71.
280. Hamman L, Hirschman I. Studies on blood sugar. Archives of Internal Medicine. 1917;20(5):761-808.
281. Staub H. Untersuchungen uber den Zuckerstoffwechsel des Munchen. Z Clin Med. 1921;91:44-8.
282. Ma J, Stevens JE, Cukier K, Maddox AF, Wishart JM, Jones KL, et al. Effects of a protein preload on gastric emptying, glycemia, gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes. Diabetes Care. 2009;32(9):1600-2.
283. Meier J, Gethmann A, Götze O, Gallwitz B, Holst JJ, Schmidt W, et al. Glucagon-like peptide 1 abolishes the postprandial rise in triglyceride concentrations and lowers levels of non-esterified fatty acids in humans. Diabetologia. 2006;49:452-8.
284. Lee S-H, Tura A, Mari A, Ko S-H, Kwon H-S, Song K-H, et al. Potentiation of the early-phase insulin response by a prior meal contributes to the second-meal phenomenon in type 2 diabetes. American Journal of Physiology-Endocrinology and Metabolism. 2011;301(5):984-90.
285. Jovanovic A, Leverton E, Solanky B, Ravikumar B, Snaar JE, Morris PG, et al. The second-meal phenomenon is associated with enhanced muscle glycogen storage in humans. Clinical Science. 2009;117(3):119-27.
286. Moore MC, Smith MS, Farmer B, Kraft G, Shiota M, Williams PE, et al. Priming Effect of a Morning Meal on Hepatic Glucose Disposition Later in the Day. Diabetes. 2017;66(5):1136-45.
287. Nieuwenhoven V. The effect of physical exercise on parameters of gastrointestinal function. Neurogastroenterology and Motility. 1999;11(6):431-9.
288. Van Wijck K, Lenaerts K, Van Loon LJ, Peters WH, Buurman WA, Dejong CH. Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PloS One. 2011;6(7):Epub: doi 10.1371/journal.pone.0022366.
289. Hall DM, Buettner GR, Oberley LW, Xu L, Matthes RD, Gisolfi CV. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. American Journal of Physiology-Heart and Circulatory Physiology. 2001;280(2):509-21.
290. Rehrer NJ, van Kemenade M, Meester W, Brouns F, Saris W. Gastrointestinal complaints in relation to dietary intake in triathletes. International Journal of Sport Nutrition. 1992;2(1):48-59.
291. Watt MJ, Howlett KF, Febbraio MA, Spriet LL, Hargreaves M. Adrenaline increases skeletal muscle glycogenolysis, pyruvate dehydrogenase activation and carbohydrate oxidation during moderate exercise in humans. The Journal of Physiology. 2001;534(1):269-78.
292. Gonzalez JT. Paradoxical second-meal phenomenon in the acute postexercise period. Nutrition. 2014;30(9):961-7.
293. Wee S-L, Williams C, Tsintzas K, Boobis L. Ingestion of a high-glycemic index meal increases muscle glycogen storage at rest but augments its utilization during subsequent exercise. Journal of Applied Physiology. 2005;99(2):707-14.

294. Perseghin G, Price TB, Petersen KF, Roden M, Cline GW, Gerow K, et al. Increased glucose transport–phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects. New England Journal of Medicine. 1996;335(18):1357-62.
295. van Dijk J-W, van Loon LJ. Exercise Strategies to Optimize Glycemic Control in Type 2 Diabetes: A Continuing Glucose Monitoring Perspective. Diabetes Spectrum. 2015;28(1):24-31.
296. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care. 1999;22(9):1462-70.
297. Stannard SR, Buckley AJ, Edge JA, Thompson MW. Adaptations to skeletal muscle with endurance exercise training in the acutely fed versus overnight-fasted state. Journal of Science and Medicine in Sport. 2010;13(4):465-9.
298. Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA, Shulman RG. Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. New England Journal of Medicine. 1990;322(4):223-8.
299. Schenk S, Horowitz JF. Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid–induced insulin resistance. The Journal of Clinical Investigation. 2007;117(6):1690-8.
300. Bergman BC, Perreault L, Strauss A, Bacon S, Kerege A, Harrison K, et al. Intramuscular triglyceride synthesis: importance in muscle lipid partitioning in humans. American Journal of Physiology-Endocrinology and Metabolism. 2017;314(2):152-64.
301. Dubé JJ, Amati F, Stefanovic-Racic M, Toledo FG, Sauers SE, Goodpaster BH. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. American Journal of Physiology-Endocrinology and Metabolism. 2008;294(5):882-8.
302. Hansen AK, Fischer CP, Plomgaard P, Andersen JL, Saltin B, Pedersen BK. Skeletal muscle adaptation: training twice every second day vs. training once daily. Journal of Applied Physiology. 2005;98(1):93-9.
303. Adamo K, Tarnopolsky M, Graham T. Dietary carbohydrate and postexercise synthesis of proglycogen and macroglycogen in human skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 1998;275(2):229-34.
304. Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA. Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. Journal of Applied Physiology. 2008;105(5):1462-70.
305. Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K, et al. Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. Medicine and Science in Sports and Exercise. 2010;42(11):2046-55.
306. Morton JP, Croft L, Bartlett JD, MacLaren DP, Reilly T, Evans L, et al. Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle. Journal of Applied Physiology. 2009;106(5):1513-21.
307. Lane SC, Camera DM, Lassiter DG, Areta JL, Bird SR, Yeo WK, et al. Effects of sleeping with reduced carbohydrate availability on acute training responses. Journal of Applied Physiology. 2015;119(6):643-55.
308. Nybo L, Pedersen K, Christensen B, Aagaard P, Brandt N, Kiens B. Impact of carbohydrate supplementation during endurance training on glycogen storage and performance. Acta Physiologica. 2009;197(2):117-27.
309. Bruce CR, Hawley JA. Improvements in insulin resistance with aerobic exercise training: a lipocentric approach. Medicine and Science in Sports and Exercise. 2004;36(7):1196-201.

310. Van Proeyen K, Szlufcik K, Nielens H, Deldicque L, Van Dyck R, Ramaekers M, et al. High-fat diet overrules the effects of training on fiber-specific intramyocellular lipid utilization during exercise. Journal of Applied Physiology. 2011;111(1):108-16.
311. Pilegaard H, Keller C, Steensberg A, Wulff Helge J, Klarlund Pedersen B, Saltin B, et al. Influence of pre-exercise muscle glycogen content on exercise-induced transcriptional regulation of metabolic genes. The Journal of Physiology. 2002;541(1):261-71.
312. Yeo WK, McGee SL, Carey AL, Paton CD, Garnham AP, Hargreaves M, et al. Acute signalling responses to intense endurance training commenced with low or normal muscle glycogen. Experimental Physiology. 2010;95(2):351-8.
313. McBride A, Ghilagaber S, Nikolaev A, Hardie DG. The Glycogen-Binding Domain on the AMPK β Subunit Allows the Kinase to Act as a Glycogen Sensor. Cell Metabolism. 2009;9(1):23-34.
314. Philp A, Hargreaves M, Baar K. More than a store: regulatory roles for glycogen in skeletal muscle adaptation to exercise. American Journal of Physiology-Endocrinology and Metabolism. 2012;302(11):1343-51.
315. Bartlett JD, Louhelainen J, Iqbal Z, Cochran AJ, Gibala MJ, Gregson W, et al. Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2013;304(6):450-8.
316. Bajaj M, Medina-Navarro R, Suraamornkul S, Meyer C, DeFronzo RA, Mandarino LJ. Paradoxical changes in muscle gene expression in insulin-resistant subjects after sustained reduction in plasma free fatty acid concentration. Diabetes. 2007;56(3):743-52.
317. Ehrenborg E, Krook A. Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor δ. Pharmacological Reviews. 2009;61(3):373-93.
318. Dressel U, Allen TL, Pippal JB, Rohde PR, Lau P, Muscat GE. The peroxisome proliferator-activated receptor β/δ agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. Molecular Endocrinology. 2003;17(12):2477-93.
319. Luquet S, Lopez-Soriano J, Holst D, Fredenrich A, Melki J, Rassoulzadegan M, et al. Peroxisome proliferator-activated receptor δ controls muscle development and oxidative capability. The FASEB Journal. 2003;17(15):2299-301.
320. Philp A, MacKenzie MG, Belew MY, Towler MC, Corstorphine A, Papalamprou A, et al. Glycogen content regulates peroxisome proliferator activated receptor-∂(PPAR-∂) activity in rat skeletal muscle. PloS One. 2013;8(10):Epub: https://doi.org/10.1371/journal.pone.0077200.
321. Handschin C, Spiegelman BM. Peroxisome proliferator-activated receptor γ coactivator 1 coactivators, energy homeostasis, and metabolism. Endocrine Reviews. 2006;27(7):728-35.
322. Cluberton LJ, McGee SL, Murphy RM, Hargreaves M. Effect of carbohydrate ingestion on exercise-induced alterations in metabolic gene expression. Journal of Applied Physiology. 2005;99(4):1359-63.
323. Andersson A, Sjödin A, Olsson R, Vessby B. Effects of physical exercise on phospholipid fatty acid composition in skeletal muscle. American Journal of Physiology-Endocrinology and Metabolism. 1998;274(3):432-8.
324. Vessby B, Tengblad S, Lithell H. Insulin sensitivity is related to the fatty acid composition of serum lipids and skeletal muscle phospholipids in 70-year-old men. Diabetologia. 1994;37(10):1044-50.
325. Helge JW, Dela F. Effect of training on muscle triacylglycerol and structural lipids: a relation to insulin sensitivity? Diabetes. 2003;52(8):1881-7.

326. Bergouignan A, Trudel G, Simon C, Chopard A, Schoeller DA, Momken I, et al. Physical inactivity differentially alters dietary oleate and palmitate trafficking. Diabetes. 2009;58(2):367-76.
327. Senoo N, Miyoshi N, Goto-Inoue N, Minami K, Yoshimura R, Morita A, et al. PGC-1α-mediated changes in phospholipid profiles of exercise-trained skeletal muscle. Journal of Lipid Research. 2015;56(12):2286-96.
328. Betts JA, Richardson JD, Chowdhury EA, Holman GD, Tsintzas K, Thompson D. The causal role of breakfast in energy balance and health: a randomized controlled trial in lean adults. The American Journal of Clinical Nutrition. 2014;100(2):539-47.
329. Chowdhury EA, Richardson JD, Holman GD, Tsintzas K, Thompson D, Betts JA. The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults. The American Journal of Clinical Nutrition. 2016;103(3):747-56.
330. Bartlett JD, Hawley JA, Morton JP. Carbohydrate availability and exercise training adaptation: too much of a good thing? European Journal of Sport Science. 2015;15(1):3-12.
331. Southgate D, Durnin J. Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. British Journal of Nutrition. 1970;24(2):517-35.
332. Beyer PL, Flynn MA. Effects of high-and low-fiber diets on human feces. Journal of the American Dietetic Association. 1978;72(3):271-7.
333. Webb P, Annis J. Adaptation to overeating in lean and overweight men and women. Clinical Nutrition. 1983;37(2):117-31.
334. Burton P, Lightowler H. The impact of freezing and toasting on the glycaemic response of white bread. European Journal of Clinical Nutrition. 2008;62(5):594-9.
335. Pennings B, Groen BB, van Dijk J-W, de Lange A, Kiskini A, Kuklinski M, et al. Minced beef is more rapidly digested and absorbed than beef steak, resulting in greater postprandial protein retention in older men. The American Journal of Clinical Nutrition. 2013;98(1):121-8.
336. McMurray RG, Soares J, Caspersen CJ, McCurdy T. Examining variations of resting metabolic rate of adults: a public health perspective. Medicine and Science in Sports and Exercise. 2014;46(7):1352-8.
337. Luke A, Schoeller D. Basal metabolic rate, fat-free mass, and body cell mass during energy restriction. Metabolism-Clinical and Experimental. 1992;41(4):450-6.
338. Westerterp KR. Diet induced thermogenesis. Nutrition and Metabolism. 2004;1(1):5.
339. Thompson D, Batterham AM, Markovitch D, Dixon NC, Lund AJ, Walhin J-P. Confusion and conflict in assessing the physical activity status of middle-aged men. PLoS One. 2009;4(2):Epub: https://doi.org/10.1371/journal.pone.0004337.
340. Turner JE, Markovitch D, Betts JA, Thompson D. Nonprescribed physical activity energy expenditure is maintained with structured exercise and implicates a compensatory increase in energy intake. The American Journal of Clinical Nutrition. 2010;92(5):1009-16.
341. Hall KD, Sacks G, Chandramohan D, Chow CC, Wang YC, Gortmaker SL, et al. Quantification of the effect of energy imbalance on bodyweight. The Lancet. 2011;378(9793):826-37.
342. Müller MJ, Enderle J, Pourhassan M, Braun W, Eggeling B, Lagerpusch M, et al. Metabolic adaptation to caloric restriction and subsequent refeeding: the Minnesota Starvation Experiment revisited. The American Journal of Clinical Nutrition. 2015;102(4):807-19.
343. Barton BA, Eldridge AL, Thompson D, Affenito SG, Striegel-Moore RH, Franko DL, et al. The relationship of breakfast and cereal consumption to nutrient intake and body mass index: the National Heart, Lung, and Blood Institute Growth and Health Study. Journal of the American Dietetic Association. 2005;105(9):1383-9.

344. Fabry P, Hejl Z, Fodor J, Braun T, Zvolánková K. The frequency of meals its relation to overweight, hypercholesterolaemia, and decreased glucose-tolerance. The Lancet. 1964;284(7360):614-5.
345. Chowdhury EA, Richardson JD, Tsintzas K, Thompson D, Betts JA. Carbohydrate-rich breakfast attenuates glycaemic, insulinaemic and ghrelin response to ad libitum lunch relative to morning fasting in lean adults. British Journal of Nutrition. 2015;114(01):98-107.
346. Thompson D, Karpe F, Lafontan M, Frayn K. Physical activity and exercise in the regulation of human adipose tissue physiology. Physiological Reviews. 2012;92(1):157-91.
347. Lahti-Koski M, Pietinen P, Heliövaara M, Vartiainen E. Associations of body mass index and obesity with physical activity, food choices, alcohol intake, and smoking in the 1982–1997 FINRISK Studies. The American Journal of Clinical Nutrition. 2002;75(5):809-17.
348. Miller WC, Koceja D, Hamilton E. A meta-analysis of the past 25 years of weight loss research using diet, exercise or diet plus exercise intervention. International Journal of Obesity. 1997;21(10):941-7.
349. King NA, Hopkins M, Caudwell P, Stubbs R, Blundell JE. Individual variability following 12 weeks of supervised exercise: identification and characterization of compensation for exercise-induced weight loss. International Journal of Obesity. 2008;32(1):177-84.
350. Williamson P, Atkinson G, Batterham A. Inter-individual differences in weight change following exercise interventions: a systematic review and meta-analysis of randomized controlled trials. Obesity Reviews. 2018;19(7):960-75.
351. Flatt JP. Macronutrient composition and food selection. Obesity Research. 2001;9(11):256-62.
352. Flatt J. The Difference in the Storage Capacities for Carbohydrate and for Fat, and Its Implications in the Regulation of Body Weight a. Annals of the New York Academy of Sciences. 1987;499(1):104-23.
353. Kissileff HR, Pi-Sunyer F, Segal K, Meltzer S, Foelsch PA. Acute effects of exercise on food intake in obese and nonobese women. The American Journal of Clinical Nutrition. 1990;52(2):240-5.
354. Hopkins M, Blundell JE, King NA. Individual variability in compensatory eating following acute exercise in overweight and obese women. British Journal of Sports Medicine. 2013;48(20):Epub: http://dx.doi.org/10.1136/bjsports-2012-091721.
355. López-Soldado I, Fuentes-Romero R, Duran J, Guinovart JJ. Effects of hepatic glycogen on food intake and glucose homeostasis are mediated by the vagus nerve in mice. Diabetologia. 2017;60(6):1076-83.
356. Robinson SL, Hattersley J, Frost GS, Chambers ES, Wallis GA. Maximal fat oxidation during exercise is positively associated with 24-hour fat oxidation and insulin sensitivity in young, healthy men. Journal of Applied Physiology. 2015;118:1415-22.
357. Marquet L-A, Brisswalter J, Louis J, Tiollier E, Burke LM, Hawley JA, et al. Enhanced Endurance Performance by Periodization of CHO Intake:“Sleep Low” Strategy. Medicine & Science in Sports & Exercise. 2016;48(4):663-72.
358. Gillen JB, Percival ME, Ludzki A, Tarnopolsky MA, Gibala MJ. Interval training in the fed or fasted state improves body composition and muscle oxidative capacity in overweight women. Obesity. 2013;21(11):2249-55.
359. Schoenfeld BJ, Aragon AA, Wilborn CD, Krieger JW, Sonmez GT. Body composition changes associated with fasted versus non-fasted aerobic exercise. Journal of the International Society of Sports Nutrition. 2014;11(1):54-63.
360. Andreoli A, Scalzo G, Masala S, Tarantino U, Guglielmi G. Body composition assessment by dual-energy X-ray absorptiometry (DXA). La Radiologia Medica. 2009;114(2):286-300.

361. Brouns F, Bjorck I, Frayn K, Gibbs A, Lang V, Slama G, et al. Glycaemic index methodology. Nutrition Research Reviews. 2005;18(1):145-71.
362. Acheson K, Schutz Y, Bessard T, Anantharaman K, Flatt J, Jequier E. Glycogen storage capacity and de novo lipogenesis during massive carbohydrate overfeeding in man. The American Journal of Clinical Nutrition. 1988;48(2):240-7.
363. Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. Medicine and Science in Sports and Exercise. 2002;34(1):92-7.
364. Borg G. Perceived exertion: a note on 'history' and methods. Medicine and Science in Sports and Exercise. 1973;5(2):90-3.
365. Taylor HL, Buskirk E, Henschel A. Maximal oxygen intake as an objective measure of cardio-respiratory performance. Journal of Applied Physiology. 1955;8(1):73-80.
366. Betts JA, Thompson D. Thinking outside the bag (not necessarily outside the lab). Medicine and Science in Sports and Exercise. 2012;44(10):2040-1.
367. Wilmore JH, Costill DL. Adequacy of the Haldane transformation in the computation of exercise V O2 in man. Journal of Applied Physiology. 1973;35(1):85-9.
368. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. The Journal of Physiology. 1949;109(1):1-9.
369. Koopman R, Pannemans DL, Jeukendrup AE, Gijsen AP, Senden JM, Halliday D, et al. Combined ingestion of protein and carbohydrate improves protein balance during ultra-endurance exercise. American Journal of Physiology Endocrinology and Metabolism. 2004;287(4):712-20.
370. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, et al. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. American Journal of Clinical Nutrition. 2009;89(1):161-8.
371. Tomé D, Bos Cc. Dietary protein and nitrogen utilization. The Journal of Nutrition. 2000;130(7):1868S-73.
372. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. American Journal of Physiology-Endocrinology and Metabolism. 2008;294(1):15-26.
373. Levy JC, Matthews DR, Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. Diabetes Care. 1998;21(12):2191-2.
374. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. The Journal of Clinical Endocrinology and Metabolism. 2000;85(7):2402-10.
375. Cederholm J, Wibell L. Insulin release and peripheral sensitivity at the oral glucose tolerance test. Diabetes Research and Clinical Practice. 1990;10(2):167-75.
376. Mari A, Pacini G, Murphy E, Ludvik B, Nolan JJ. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. Diabetes Care. 2001;24(3):539-48.
377. Gonzalez J, Barwood M, Goodall S, Thomas K, Howatson G. Alterations in whole-body insulin sensitivity resulting from repeated eccentric exercise of a single muscle group: a pilot investigation. International Journal of Sport Nutrition and Exercise Metabolism. 2015;25(4):405-10.
378. Hall R. Vascular injuries resulting from arterial puncture or catheterization. British Journal of Surgery. 1971;58(7):513-6.
379. Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. Metabolism. 1981;30(9):936-40.

380. Brooks DC, Black PR, Arcangeli MA, Aoki TT, Wilmore DW. The heated dorsal hand vein: an alternative arterial sampling site. Journal of Parenteral and Enteral Nutrition. 1989;13(1):102-5.
381. Liu D, Moberg E, Kollind M, Lins P-E, Adamson U, Macdonald I. Arterial, arterialized venous, venous and capillary blood glucose measurements in normal man during hyperinsulinaemic euglycaemia and hypoglycaemia. Diabetologia. 1992;35(3):287-90.
382. McGuire E, Helderman J, Tobin J, Andres R, Berman M. Effects of arterial versus venous sampling on analysis of glucose kinetics in man. Journal of Applied Physiology. 1976;41(4):565-73.
383. Nauck M, Liess H, Siegel E, Niedmann P, Creutzfeldt W. Critical evaluation of the 'heated-hand-technique'for obtaining 'arterialized'venous blood: incomplete arterialization and alterations in glucagon responses. Clinical Physiology. 1992;12(5):537-52.
384. Jensen MD, Heiling VJ. Heated hand vein blood is satisfactory for measurements during free fatty acid kinetic studies. Metabolism. 1991;40(4):406-9.
385. Sonnenberg G, Keller U. Sampling of arterialized heated-hand venous blood as a noninvasive technique for the study of ketone body kinetics in man. Metabolism. 1982;31(1):1-5.
386. Reichard GA, Moury NF, Hochella NJ, Patterson A, Weinhouse S. Quantitative estimation of the Cori cycle in the human. Journal of Biological Chemistry. 1963;238(2):495-501.
387. Ridley W, Houchins J, Kirkwood S. Mechanism of action of uridine diphosphoglucose dehydrogenase. Evidence for a second reversible dehydrogenation step involving an essential thiol group. Journal of Biological Chemistry. 1975;250(22):8761-7.
388. Wajngot A, Chandramouli V, Schumann WC, Kumaran K, Efendic S, Landau BR. Testing of the assumptions made in estimating the extent of futile cycling. American Journal of Physiology-Endocrinology and Metabolism. 1989;256(5):668-75.
389. Tigas SK, Sunehag AL, Haymond MW. Impact of duration of infusion and choice of isotope label on isotope recycling in glucose homeostasis. Diabetes. 2002;51(11):3170-5.
390. Breen L, Philp A, Shaw CS, Jeukendrup AE, Baar K, Tipton KD. Beneficial effects of resistance exercise on glycemic control are not further improved by protein ingestion. PLoS One. 2011;6(6):Epub: https://doi.org/10.1371/journal.pone.0020613.
391. Ahlborg G, Felig P, Hagenfeldt L, Hendler R, Wahren J. Substrate turnover during prolonged exercise in man: splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. Journal of Clinical Investigation. 1974;53(4):1080-90.
392. Jentjens U, Achten, Currell, Mann, and Jeukendrup. Exogenous carbohydrate oxidation rates are elevated after combined ingestion of glucose and fructose during exercise in the heat. Journal of Applied Physiology. 2006;100(3):807-16.
393. Wolfe RR. Radioactive and stable isotope tracers in biomedicine: principles and practice of kinetic analysis. 1992.
394. Radziuk J. An integral equation approach to measuring turnover in nonsteady compartmental and distributed systems. Bulletin of Mathematical Biology. 1976;38(6):679-93.
395. Radziuk J, Norwich KH, Vranic M. Experimental validation of measurements of glucose turnover in nonsteady state. American Journal of Physiology-Endocrinology and Metabolism. 1978;234(1):E84.
396. Steele R, Wall J, De Bodo R, Altszuler N. Measurement of size and turnover rate of body glucose pool by the isotope dilution method. American Journal of Physiology-Legacy Content. 1956;187(1):15-24.

397. Steele R, Bjerknes C, Rathgeb I, Altszuler N. Glucose uptake and production during the oral glucose tolerance test. Diabetes. 1968;17(7):415-21.
398. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. Annals of the New York Academy of Sciences. 1959;82(1):420-30.
399. Rizza RA, Toffolo G, Cobelli C. Accurate Measurement of Postprandial Glucose Turnover: Why Is It Difficult and How Can It Be Done (Relatively) Simply? Diabetes. 2016;65(5):1133-45.
400. Basu R, Di Camillo B, Toffolo G, Basu A, Shah P, Vella A, et al. Use of a novel triple-tracer approach to assess postprandial glucose metabolism. American Journal of Physiology-Endocrinology and Metabolism. 2003;284(1):55-69.
401. Livesey G, Wilson P, Dainty J, Brown J, Faulks R, Roe M, et al. Simultaneous time-varying systemic appearance of oral and hepatic glucose in adults monitored with stable isotopes. American Journal of Physiology-Endocrinology and Metabolism. 1998;275(4):717-28.
402. Tarnopolsky MA, Pearce E, Smith K, Lach B. Suction-modified Bergström muscle biopsy technique: Experience with 13,500 procedures. Muscle & Nerve. 2011;43(5):716-25.
403. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences. 1979;76(9):4350-4.
404. Deighton K, Frampton J, Gonzalez J. Test-meal palatability is associated with overconsumption but better represents preceding changes in appetite in non-obese males. British Journal of Nutrition. 2016;116(5):935-43.
405. Whybrow S, Horgan G, Stubbs R. Low-energy reporting and duration of recording period. European Journal of Clinical Nutrition. 2008;62(9):1148-50.
406. Morgan MY, Levine JA. Alcohol and nutrition. Proceedings of the Nutrition Society. 1988;47(2):85-98.
407. Stubbs RJ, O'Reilly LM, Whybrow S, Fuller Z, Johnstone AM, Livingstone MBE, et al. Measuring the difference between actual and reported food intakes in the context of energy balance under laboratory conditions. British Journal of Nutrition. 2014;111(11):2032-43.
408. Robinson E, Hardman CA, Halford JC, Jones A. Eating under observation: a systematic review and meta-analysis of the effect that heightened awareness of observation has on laboratory measured energy intake, 2. The American Journal of Clinical Nutrition. 2015;102(2):324-37.
409. Livingstone MBE, Black AE. Markers of the validity of reported energy intake. The Journal of Nutrition. 2003;133(3):895-920.
410. Jenab M, Slimani N, Bictash M, Ferrari P, Bingham SA. Biomarkers in nutritional epidemiology: applications, needs and new horizons. Human Genetics. 2009;125(5-6):507-25.
411. Compher C, Frankenfield D, Keim N, Roth-Yousey L, Group EAW. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. Journal of the American Dietetic Association. 2006;106(6):881-903.
412. Brage S, Ekelund U, Brage N, Hennings MA, Froberg K, Franks PW, et al. Hierarchy of individual calibration levels for heart rate and accelerometry to measure physical activity. Journal of Applied Physiology. 2007;103(2):682-92.
413. Brage S, Westgate K, Franks PW, Stegle O, Wright A, Ekelund U, et al. Estimation of free-living energy expenditure by heart rate and movement sensing: a doubly-labelled water study. PloS one. 2015;10(9):Epub: https://doi.org/10.1371/journal.pone.0137206.
414. Kenny GP, Notley SR, Gagnon D. Direct calorimetry: a brief historical review of its use in the study of human metabolism and thermoregulation. European journal of applied physiology. 2017;117(9):1765-85.

415. Levine JA. Measurement of energy expenditure. Public Health Nutrition. 2005;8(7):1123-32.
416. Douglas CG. A method for determinng the total respiratory exchange in man. The Journal of Physiology. 1911;42(1):1-2.
417. Westerterp KR. Doubly labelled water assessment of energy expenditure: principle, practice, and promise. European journal of applied physiology. 2017;117(7):1277-85.
418. Jeran S, Steinbrecher A, Pischon T. Prediction of activity-related energy expenditure using accelerometer-derived physical activity under free-living conditions: a systematic review. International Journal of Obesity. 2016;40(8):1187-97.
419. Bot S, Hollander A. The relationship between heart rate and oxygen uptake during non-steady state exercise. Ergonomics. 2000;43(10):1578-92.
420. Brage S, Brage N, Franks PW, Ekelund U, Wong M-Y, Andersen LB, et al. Branched equation modeling of simultaneous accelerometry and heart rate monitoring improves estimate of directly measured physical activity energy expenditure. Journal of Applied Physiology. 2004;96(1):343-51.
421. Brage S, Brage N, Franks P, Ekelund U, Wareham N. Reliability and validity of the combined heart rate and movement sensor Actiheart. European Journal of Clinical Nutrition. 2005;59(4):561-70.
422. Wolever TM. Effect of blood sampling schedule and method of calculating the area under the curve on validity and precision of glycaemic index values. British Journal of Nutrition. 2004;91(2):295-300.
423. Maxwell SE, Delaney HD. Designing experiments and analyzing data : a model comparison perspective. Wadsworth.: Belmont, Ca; 1990.
424. Atkinson G. Analysis of repeated measurements in physical therapy research: multiple comparisons amongst level means and multi-factorial designs. Physical Therapy in Sport. 2002;3(4):191-203.
425. Loftus GR, Masson MEJ. Using confidence intervals in within-subject designs. Psychonomic Bulletin & Review. 1994;1(4):476-90.
426. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. The Journal of Physiology. 2010;588(23):4795-810.
427. Hawley JA, Lundby C, Cotter JD, Burke LM. Maximizing cellular adaptation to endurance exercise in skeletal muscle. Cell Metabolism. 2018;27(5):962-76.
428. Goldschmidt S, Light AB. A method of obtaining from veins blood similar to arterial blood in gaseous content. Journal of Biological Chemistry. 1925;64(1):53-8.
429. Glassberg B. The arteriovenous difference in blood sugar content. Archives of Internal Medicine. 1930;46(4):605-9.
430. Rabinowitch I. Simultaneous determinations of arterial and venous blood-sugars in diabetic individuals. British Journal Experimental Pathology. 1927;8:76-84.
431. Devlin JT, Barlow J, Horton ES. Whole body and regional fuel metabolism during early postexercise recovery. American Journal of Physiology-Endocrinology And Metabolism. 1989;256(1):167-72.
432. Edinburgh R, Betts J, Thompson D, Gonzalez J. Physiologists, watch where you place that cannula! Physiology News. 2017;109:20-3.
433. Waterson MJ, Horvath TL. Neuronal regulation of energy homeostasis: beyond the hypothalamus and feeding. Cell Metabolism. 2015;22(6):962-70.
434. Timper K, Brüning JC. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. Disease Models and Mechanisms. 2017;10:679-89.
435. Wilson JL, Enriori PJ. A talk between fat tissue, gut, pancreas and brain to control body weight. Molecular and Cellular Endocrinology. 2015;418(1):108-19.

436. Geliebter A. Gastric distension and gastric capacity in relation to food intake in humans. Physiology and Behavior. 1988;44(4):665-8.
437. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 2001;50(8):1714-9.
438. Natalucci G, Riedl S, Gleiss A, Zidek T, Frisch H. Spontaneous 24-h ghrelin secretion pattern in fasting subjects: maintenance of a meal-related pattern. European Journal of Endocrinology. 2005;152(6):845-50.
439. Wren A, Seal L, Cohen M, Brynes A, Frost G, Murphy K, et al. Ghrelin enhances appetite and increases food intake in humans. The Journal of Clinical Endocrinology and Metabolism. 2001;86(12):5992-3.
440. Garin MC, Burns CM, Kaul S, Cappola AR. The human experience with ghrelin administration. The Journal of Clinical Endocrinology and Metabolism. 2013;98(5):1826-37.
441. Chaudhri O, Small C, Bloom S. Gastrointestinal hormones regulating appetite. Philosophical Transactions of the Royal Society of London B: Biological Sciences. 2006;361(1471):1187-209.
442. Chambers L, McCrickerd K, Yeomans MR. Optimising foods for satiety. Trends in Food Science and Technology. 2015;41(2):149-60.
443. Lean M, Malkova D. Altered gut and adipose tissue hormones in overweight and obese individuals: cause or consequence? International Journal of Obesity. 2016;40(4):622-32.
444. Reseland JE, Anderssen SA, Solvoll K, Hjermann I, Urdal P, Holme I, et al. Effect of long-term changes in diet and exercise on plasma leptin concentrations–. The American Journal of Clinical Nutrition. 2001;73(2):240-5.
445. Thong FS, Hudson R, Ross R, Janssen I, Graham TE. Plasma leptin in moderately obese men: independent effects of weight loss and aerobic exercise. American Journal of Physiology-Endocrinology and Metabolism. 2000;279:307-13.
446. Morton G, Cummings D, Baskin D, Barsh G, Schwartz M. Central nervous system control of food intake and body weight. Nature. 2006;443(7109):289-95.
447. Seufert J, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C, et al. Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. The Journal of Clinical Endocrinology and Metabolism. 1999;84(2):670-6.
448. Rosenbaum M, Sy M, Pavlovich K, Leibel RL, Hirsch J. Leptin reverses weight loss–induced changes in regional neural activity responses to visual food stimuli. The Journal of Clinical Investigation. 2008;118(7):2583-91.
449. Mayer J. Glucostatic mechanism of regulation of food intake. New England Journal of Medicine. 1953;249(1):13-6.
450. Pannacciulli N, Salbe AD, Ortega E, Venti CA, Bogardus C, Krakoff J. The 24-h carbohydrate oxidation rate in a human respiratory chamber predicts ad libitum food intake–. The American Journal of Clinical Nutrition. 2007;86(3):625-32.
451. Long SJ, Hart K, Morgan LM. The ability of habitual exercise to influence appetite and food intake in response to high-and low-energy preloads in man. British Journal of Nutrition. 2002;87(5):517-23.
452. Van Walleghen EL, Orr JS, Gentile CL, Davy KP, Davy BM. Habitual physical activity differentially affects acute and short-term energy intake regulation in young and older adults. International Journal Of Obesity. 2007;31(8):1277-85.
453. Alméras N, Lavallée N, Després J-P, Bouchard C, Tremblay A. Exercise and energy intake: effect of substrate oxidation. Physiology and Behavior. 1995;57(5):995-1000.
454. Jensen TE, Richter EA. Regulation of glucose and glycogen metabolism during and after exercise. The Journal of Physiology. 2012;590(5):1069-76.

455. Xue B, Kahn BB. AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues. The Journal of Physiology. 2006;574(1):73-83.
456. Friedrichsen M, Mortensen B, Pehmøller C, Birk JB, Wojtaszewski JF. Exercise-induced AMPK activity in skeletal muscle: role in glucose uptake and insulin sensitivity. Molecular and Cellular Endocrinology. 2013;366(2):204-14.
457. Kola B. Role of AMP-Activated Protein Kinase in the Control of Appetite. Journal of Neuroendocrinology. 2008;20(7):942-51.
458. Minokoshi Y, Alquier T, Furukawa N, Kim Y-B, Lee A, Xue B, et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature. 2004;428:569.
459. Andersson U, Treebak JT, Nielsen JN, Smith KL, Abbott CR, Small CJ, et al. Exercise in rats does not alter hypothalamic AMP-activated protein kinase activity. Biochemical and biophysical research communications. 2005;329(2):719-25.
460. Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B, et al. Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. Journal of Physiology. 2001;537(Pt 2):633-9.
461. Pedersen BK, Steensberg A, Fischer C, Keller C, Keller P, Plomgaard P, et al. Searching for the exercise factor: is IL-6 a candidate? Journal of Muscle Research & Cell Motility. 2003;24(2):113-20.
462. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nature Medicine. 2002;8(1):75-9.
463. Wedell-Neergaard A-S, Lehrskov LL, Christensen RH, Legaard GE, Dorph E, Larsen MK, et al. Exercise-induced changes in visceral adipose tissue mass are regulated by IL-6 signaling: a randomized controlled trial. Cell Metabolism. 2018;29(4):844-55.
464. Islam H, Townsend LK, McKie GL, Medeiros PJ, Gurd BJ, Hazell TJ. Potential involvement of lactate and interleukin-6 in the appetite-regulatory hormonal response to an acute exercise bout. Journal of Applied Physiology. 2017;123(3):614-23.
465. Snitker S, Larson DE, Tataranni PA, Ravussin E. Ad libitum food intake in humans after manipulation of glycogen stores. The American Journal of Clinical Nutrition. 1997;65(4):941-6.
466. Shetty PS, Prentice AM, Goldberg GR, Murgatroyd PR, McKenna A, Stubbs RJ, et al. Alterations in fuel selection and voluntary food intake in response to isoenergetic manipulation of glycogen stores in humans. The American Journal of Clinical Nutrition. 1994;60(4):534-43.
467. Stubbs RJ, Murgatroyd PR, Goldberg GR, Prentice AM. Carbohydrate balance and the regulation of day-to-day food intake in humans. The American Journal of Clinical Nutrition. 1993;57(6):897-903.
468. Gamberucci A, Marcolongo P, Fulceri R, Giunti R, Watkins SL, Waddell ID, et al. Low levels of glucose-6-phosphate hydrolysis in the sarcoplasmic reticulum of skeletal muscle: involvement of glucose-6-phosphatase. Molecular Membrane Biology. 1996;13(2):103-8.
469. López-Soldado I, Zafra D, Duran J, Adrover A, Calbó J, Guinovart JJ. Liver Glycogen Reduces Food Intake and Attenuates Obesity in a High-Fat Diet–Fed Mouse Model. Diabetes. 2015;64(3):796-807.
470. Allen M, Bradford B, Oba M. Board-Invited Review: The hepatic oxidation theory of the control of feed intake and its application to ruminants. Journal of Animal Science. 2009;87(10):3317-34.
471. von Holstein-Rathlou S, BonDurant LD, Peltekian L, Naber MC, Yin TC, Claflin KE, et al. FGF21 mediates endocrine control of simple sugar intake and sweet taste preference by the liver. Cell Metabolism. 2016;23(2):335-43.

472. Hall KD, Chen KY, Guo J, Lam YY, Leibel RL, Mayer LE, et al. Energy expenditure and body composition changes after an isocaloric ketogenic diet in overweight and obese men. The American Journal of Clinical Nutrition. 2016;104(2):324-33.
473. Koutsari C, Karpe F, Humphreys S, Frayn K, Hardman A. Plasma leptin is influenced by diet composition and exercise. International Journal of Obesity. 2003;27(8):901-6.
474. AL Mulla N, Simonsen L, Bülow J. Post-exercise adipose tissue and skeletal muscle lipid metabolism in humans: the effects of exercise intensity. The Journal of Physiology. 2000;524(3):919-28.
475. Sonnenberg GE, Krakower GR, Hoffmann RG, Maas DL, Hennes MM, Kissebah AH. Plasma leptin concentrations during extended fasting and graded glucose infusions: relationships with changes in glucose, insulin, and FFA. The Journal of Clinical Endocrinology and Metabolism. 2001;86(10):4895-900.
476. Watt MJ, Holmes AG, Pinnamaneni SK, Garnham AP, Steinberg GR, Kemp BE, et al. Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. American Journal of Physiology-Endocrinology and Metabolism. 2006;290(3):E500-E8.
477. Szkudelski T. Intracellular mediators in regulation of leptin secretion from adipocytes. Physiological research. 2007;56(5):503-12.
478. Kolaczynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ, Henry R, et al. Acute and chronic effect of insulin on leptin production in humans: studies in vivo and in vitro. Diabetes. 1996;45(5):699-701.
479. Bessesen DH, Bull S, Cornier MA. Trafficking of dietary fat and resistance to obesity. Physiology and Behavior. 2008;94(5):681-8.
480. Jeukendrup AE, Wagenmakers AJM, Stegen JHCH, Gijsen AP, Brouns F, Saris WHM. Carbohydrate ingestion can completely suppress endogenous glucose production during exercise. American Journal of Physiology-Endocrinology and Metabolism. 1999;276(4):672-83.
481. Gonzalez JT, Betts JA. Dietary sugars, exercise and hepatic carbohydrate metabolism. Proceedings of the Nutrition Society. 2018:1-11.
482. Mikkelsen KH, Seifert T, Secher NH, Grøndal T, van Hall G. Systemic, cerebral and skeletal muscle ketone body and energy metabolism during acute hyper-D-β-hydroxybutyratemia in post-absorptive healthy males. The Journal of Clinical Endocrinology and Metabolism. 2015;100(2):636-43.
483. Miller BF, Fattor JA, Jacobs KA, Horning MA, Navazio F, Lindinger MI, et al. Lactate and glucose interactions during rest and exercise in men: effect of exogenous lactate infusion. The Journal of Physiology. 2002;544(3):963-75.
484. Taylor R, Price TB, Rothman DL, Shulman RG, Shulman GI. Validation of 13C NMR measurement of human skeletal muscle glycogen by direct biochemical assay of needle biopsy samples. Magnetic Resonance in Medicine. 1992;27(1):13-20.
485. Gruetter R, Magnusson I, Rothman DL, Avison MJ, Shulman RG, Shulman GI. Validation of 13C NMR measurements of liver glycogen in vivo. Magnetic Resonance in Medicine. 1994;31(6):583-8.
486. Fuchs CJ, Gonzalez JT, Beelen M, Cermak NM, Smith FE, Thelwall PE, et al. Sucrose ingestion after exhaustive exercise accelerates liver, but not muscle glycogen repletion compared with glucose ingestion in trained athletes. Journal of Applied Physiology. 2016;120(11):1328-34.
487. Hutber CA, Bonen A. Glycogenesis in muscle and liver during exercise. Journal of Applied Physiology. 1989;66(6):2811-7.
488. Ross R, Dagnone D, Jones P, Smith H, Paddags A, Hudson R. The Effects of Losing Weight through Diet or Exercise Programs on Obesity. Annals of Internal Medicine. 2000;133(2):92-3.

489. Coker RH, Williams RH, Yeo SE, Kortebein PM, Bodenner DL, Kern PA, et al. The impact of exercise training compared to caloric restriction on hepatic and peripheral insulin resistance in obesity. The Journal of Clinical Endocrinology and Metabolism. 2009;94(11):4258-66.
490. Burke LM, Hawley JA. Swifter, higher, stronger: What's on the menu? Science. 2018;362(6416):781-7.
491. Gonzalez JT, Richardson JD, Chowdhury EA, Koumanov F, Holman GD, Cooper S, et al. Molecular adaptations of adipose tissue to 6 weeks of morning fasting vs. daily breakfast consumption in lean and obese adults. The Journal of Physiology. 2018;596(4):609-22.
492. Cartee GD. Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. Diabetologia. 2015;58(1):19-30.
493. Herzig S, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. Nature Reviews Molecular Cell Biology. 2018;19(2):121-35.
494. Impey SG, Hammond KM, Shepherd SO, Sharples AP, Stewart C, Limb M, et al. Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes. Physiological Reports. 2016;4(10):Epub: https://doi:10.14814/phy2.2803.
495. Taylor R, Price TB, Katz L, Shulman RG, Shulman GI. Direct measurement of change in muscle glycogen concentration after a mixed meal in normal subjects. American Journal of Physiology-Endocrinology and Metabolism. 1993;265(2):224-9.
496. Carey PE, Halliday J, Snaar J, Morris P, Taylor R. Direct assessment of muscle glycogen storage after mixed meals in normal and type 2 diabetic subjects. American Journal of Physiology-Endocrinology and Metabolism. 2003;284(4):E688-E94.
497. Hopkins M. Does Hepatic Carbohydrate Availability Influence Postexercise Compensation in Energy Intake? The Journal of Nutrition. 2019;149(8):1305-6.
498. Hall KD, Heymsfield SB, Kemnitz JW, Klein S, Schoeller DA, Speakman JR. Energy balance and its components: implications for body weight regulation–. The American Journal of Clinical Nutrition. 2012;95(4):989-94.
499. Blundell J, King NA. Exercise, appetite control, and energy balance. Nutrition. 2000;16(7):519-22.
500. Atherton P, Smith K. Muscle protein synthesis in response to nutrition and exercise. The Journal of Physiology. 2012;590(5):1049-57.
501. Felig P. Amino acid metabolism in man. Annual Review of Biochemistry. 1975;44(1):933-55.
502. Vendelbo MH, Møller AB, Christensen B, Nellemann B, Clasen BFF, Nair KS, et al. Fasting increases human skeletal muscle net phenylalanine release and this is associated with decreased mTOR signaling. PLoS One. 2014;9(7):Epub: http://doi:10.1371/journal.pone.0102031.
503. Kumar V, Atherton P, Smith K, Rennie MJ. Human muscle protein synthesis and breakdown during and after exercise. Journal of Applied Physiology. 2009;106(6):2026-39.
504. Tipton KD, Elliott TA, Cree MG, Aarsland AA, Sanford AP, Wolfe RR. Stimulation of net muscle protein synthesis by whey protein ingestion before and after exercise. American Journal of Physiology-Endocrinology and Metabolism. 2007;292(1):71-6.
505. Acheson KJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Emady-Azar S, Ammon-Zufferey C, et al. Protein choices targeting thermogenesis and metabolism–. The American Journal of Clinical Nutrition. 2011;93(3):525-34.
506. Taylor C, Bartlett JD, van de Graaf CS, Louhelainen J, Coyne V, Iqbal Z, et al. Protein ingestion does not impair exercise-induced AMPK signalling when in a glycogen-depleted state: implications for train-low compete-high. European journal of applied physiology. 2013;113(6):1457-68.

507. Impey SG, Smith D, Robinson AL, Owens DJ, Bartlett JD, Smith K, et al. Leucine-enriched protein feeding does not impair exercise-induced free fatty acid availability and lipid oxidation: beneficial implications for training in carbohydrate-restricted states. Amino Acids. 2015;47(2):407-16.
508. Dangin M, Boirie Y, Garcia-Rodenas C, Gachon P, Fauquant J, Callier P, et al. The digestion rate of protein is an independent regulating factor of postprandial protein retention. American Journal of Physiology-Endocrinology and Metabolism. 2001;280(2):340-8.
509. Andersson-Hall U, Pettersson S, Edin F, Pedersen A, Malmodin D, Madsen K. Metabolism and Whole-Body Fat Oxidation Following Postexercise Carbohydrate or Protein Intake. International Journal of Sport Nutrition and Exercise Metabolism. 2018;28(1):37-45.
510. Sato S, Basse AL, Schönke M, Chen S, Samad M, Altıntaş A, et al. Time of Exercise Specifies the Impact on Muscle Metabolic Pathways and Systemic Energy Homeostasis. Cell Metabolism. 2019;30:1-19.
511. Dyar KA, Lutter D, Artati A, Ceglia NJ, Liu Y, Armenta D, et al. Atlas of circadian metabolism reveals system-wide coordination and communication between clocks. Cell. 2018;174(6):1571-85.
512. Asher G, Sassone-Corsi P. Time for food: the intimate interplay between nutrition, metabolism, and the circadian clock. Cell. 2015;161(1):84-92.
513. Tognini P, Murakami M, Liu Y, Eckel-Mahan KL, Newman JC, Verdin E, et al. Distinct circadian signatures in liver and gut clocks revealed by ketogenic diet. Cell metabolism. 2017;26(3):523-38. e5.
514. Savikj M, Gabriel BM, Alm PS, Smith J, Caidahl K, Björnholm M, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. Diabetologia. 2019;62(2):233-7.
515. Gill JM, Herd SL, Vora V, Hardman AE. Effects of a brisk walk on lipoprotein lipase activity and plasma triglyceride concentrations in the fasted and postprandial states. European journal of applied physiology. 2003;89(2):184-90.
516. Herd SL, Kiens B, Boobis LH, Hardman AE. Moderate exercise, postprandial lipemia, and skeletal muscle lipoprotein lipase activity. Metabolism-Clinical and Experimental. 2001;50(7):756-62.
517. Randell RK, Rollo I, Roberts TJ, Dalrymple KJ, Jeukendrup AE, Carter JM. Maximal fat oxidation rates in an athletic population. Medicine and Science in Sports and Exercise. 2017;49(1):133-40.
518. Cheneviere X, Borrani F, Sangsue D, Gojanovic B, Malatesta D. Gender differences in whole-body fat oxidation kinetics during exercise. Applied Physiology, Nutrition and Metabolism. 2011;36(1):88-95.
519. Tarnopolsky MA. Gender differences in substrate metabolism during endurance exercise. Canadian Journal of Applied Physiology. 2000;25(4):312-27.
520. Geer EB, Shen W. Gender differences in insulin resistance, body composition, and energy balance. Gender Medicine. 2009;6:60-75.